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Age-dependent alterations in spermatogenesis in *itchy* mice

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Age-dependent alteration in spermatogenesis in *itchy* mice

by

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Dedication

For my mom, who taught me that a B was never good enough; for the Army, which paid for every penny of my degrees (hooah); but most of all for me, because I worked my "testes" off to get where I am today.

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Age-dependent alteration in spermatogenesis in *itchy* mice

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Spermatogenesis is an intricate process that strongly depends on the rapid turnover of short-lived proteins, both in the differentiating germ cells and in the supportive Sertoli cells. Recent evidence has demonstrated the importance of the ubiquitin-proteasome system for this turnover, with the final enzymatic E3 ligase providing the target specificity. One E3 ligase, Itch, has been well characterized in the immune system, but its role during spermatogenesis is not yet well understood. Mice lacking functional Itch protein display a late onset autoimmune disease characterized by severe inflammation, infiltration of immune cells into various organs, and most apparently chronic dermatitis, ultimately dying from pulmonary inflammation at 6 to 9 months of age. The work presented here evaluates the testes of *itchy* mice at two developmental time points, during the peri-pubertal period at postnatal day (PND) 28 and at adulthood, PND 56. Itchy mice are smaller in size and have lower spermatid head counts, most likely resulting from an increase in germ cell apoptosis rather than a decrease in Sertoli cell number. Litter sizes are reduced in the homozygous itchy colonies, with data suggesting a defect during fetal development and not in gamete production, although survival rates tend to be similar to that of wild type. At PND 28, itchy mice show a delay in spermatogenesis and an increase in meiotic figures, while PND 56 mice show alterations in germ cell layers, spermatid head formation, and

irregular cell division. Examination of the previously identified targets of Itch revealed no significant increases in the testis, but led to discovery of immunoglobulin (IgG) deposits within the interstitial space. Changes in protein expression outside of the seminiferous epithelium suggest that cells of the immune system may be influencing proper development and functional spermatogenesis in the testis. While the previous studies using the itchy mice focused primarily on the late onset autoimmune dysfunction in these animals, increased spleen weights and changes in testicular protein are observed as early as PND 28, indicating that the loss of Itch impacts these animals much earlier during development. Taken together, these data indicate that Itch is required for functional spermatogenesis and that it may play different cellular roles depending on the developmental age of the animal. Future work is targeted at identifying the possible testis-specific targets of Itch and deciphering whether the observed phenotypes are the result of the primary loss of Itch or are a secondary effect from the overactive immune system.

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List of Abbreviations

a^{18H}	*nonagouti-lethal 18H*
ABC	avidin/biotin complex
ADP	adenosine diphosphate
Apaf1	apoptosis protease activating factor 1
ATP	adenosine triphosphate
BTB	blood testis barrier
Caspase	cysteine aspartate protease
cFLIP	cellular FLICE-like inhibitory protein
CUL4A	Cullin-4A
DAB	3,3'-diaminobenzidine
DISC	death inducing signaling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DR	death receptor
ERK	extraceullar signal-regulated kinase
FADD	Fas-associated death domain
Fas	Fas receptor
FasL	Fas ligand
HBSS	Hank's balanced salt soltuion
HECT	homologous to E6-AP carboxy terminus
IAP	inhibitor of apoptosis protein
IgG	immunoglobulin G
JNK	c-Jun N-terminal Kinase

LHR	luteinizing hormone receptor
MEHP	mono-(2-ethylhexyl) phthalate
NIH	National Institute of Health
PAS-H	periodic acid-Schiff-hematoxylin
PCR	polymerase chain reaction
p-ERK	phosphorylated ERK
PND	postnatal day
PVDF	polyvinylidene difluoride membrane
RING	really interesting new gene
RIPA	radio-immunoprecipitation assay
RNA	ribonucleic acid
ROS	reactive oxygen species
SEM	standard error of the mean
SOD1	superoxide dismutase 1
T_h cells	T helper cells
TNF	tumor necrosis factor
TNFR	TNF receptor
TRAIL	TNF-related apoptosis inducing Ligand
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
USP2	ubiquitin specific peptidase
XIAP	X-linked inhibitor of apoptosis

Chapter 1: Introduction

1.1 Overview of the Testis

1.1.1 Testis Development and Structure

Testis development begins from an otherwise sexually indifferent population of germ cells, which under the direction of the *sry* gene located on the y chromosome, differentiate into the male gonad beginning around embryonic day 10.5[1, 2]. Cords begin to form on embryonic day 12.5, separating the various cell types and distinguishing the fetal testis from the similarly-aged ovary[3-6]. Within the developed testis, highly coiled "spaghetti-like" structures called seminiferous tubules repeatedly lap the length of the organ, ultimately funneling together and exiting at a common location called the rete testis (Figure 1A). Sperm cells made in these seminiferous tubules travel from the testis, through the epididymis, which acts as a storage site, and eventually out of the body. Surrounding each tubule is a layer of peritubular myoid cells, which serve to isolate the seminiferous tubules from the interstitial space and provide the contractile motion that allows the sperm to move throughout the tubules[7, 8] (Figure 1B). Found in the space between the tubules (Figure 1B) are the testosterone-producing Leydig cells, a vital male hormone required throughout male development that is made in the testis and distributed to the rest of the body. Also in the interstitial space are blood vessels and various immune cells, including lymphocytes, resident macrophages and, in the case of infection or inflammation, infiltrating macrophages.

There are two major cell types within the seminiferous epithelium: the developing germ cells and the supportive somatic Sertoli cells (Figure 2A). The Sertoli cells extend from the basement membrane of the tubule to the lumen, and in the mouse, each Sertoli cell is in contact with and capable of supporting 30-50 germ cells[9, 10]. Although it varies

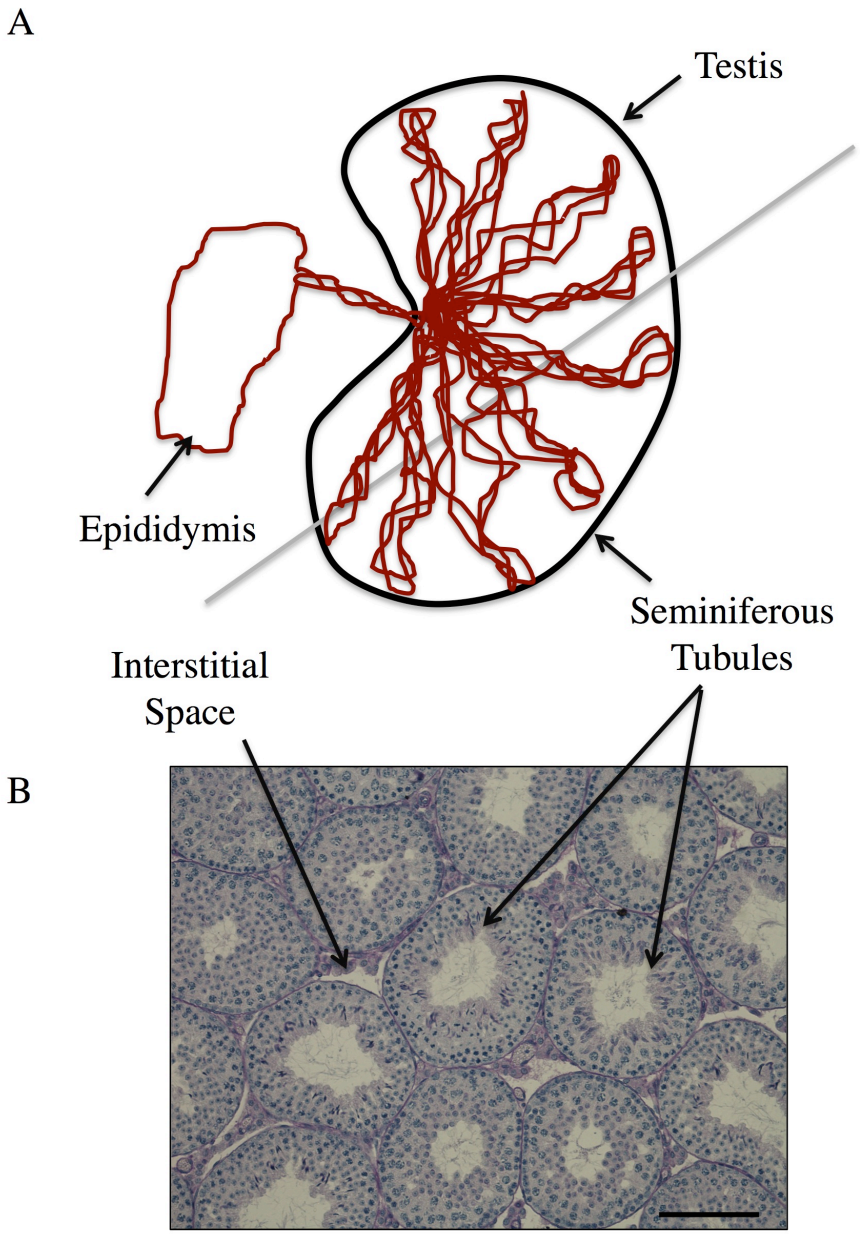

Figure 1.1 Diagram of the testis and cross section of PND 28 C57BL/6J mouse testis.

A) Diagram representing the gross structure of the testis and epididymis and B) a testicular cross section from a PND 28 C57BL/6J wild type mouse stained with PAS-H (periodic acid-Schiff-hematoxylin). Bar represents 100μm.

by species, rodent Sertoli cells become terminally differentiated early on during the neonatal period, around two weeks of age, and therefore stop dividing before the animal reaches puberty[11]. This becomes significant for testis size, as studies in which the numbers of Sertoli cells have been altered have shown that it is ultimately the number of Sertoli cells that defines the number of spermatids produced[12]. Being the only somatic cell within the seminiferous epithelium, Sertoli cells are responsible for providing nutritional, structural, and signaling support to the developing germ cells. At puberty, Sertoli cells form junctional connections with neighboring Sertoli cells, two distinct compartments, the basal compartment and the adluminal compartment, which is necessary for multiple aspects of spermatogenesis including fluid dynamics, lumen formation, and immune privilege[11, 13]. The other major cell type that resides within the seminiferous tubules is the germ cells, which divide and differentiate to form the male gamete sperm cells. The most undifferentiated germ cells, called spermatogonia, reside at the basement membrane, while the most differentiated germ cells, the elongate spermatids, are located along the lumen where they will ultimately be released (Figure 2A).

1.1.2 Spermatogenesis and tubule staging

Spermatogenesis is the process in which diploid spermatogonial stem cells divide and differentiate while moving through the seminiferous epithelium, ultimately being released into the lumen as fully mature haploid spermatids (Figure 2B). The earliest of these stem germ cells must go through two types of divisions: a self-renewal division to replenish the population of stem germ cells and early spermatogonia[14, 15]. The spermatogonia go through several rounds of DNA synthesis and form the next layer of germ cells, called spermatocytes. These large cells undergo two meiotic divisions to

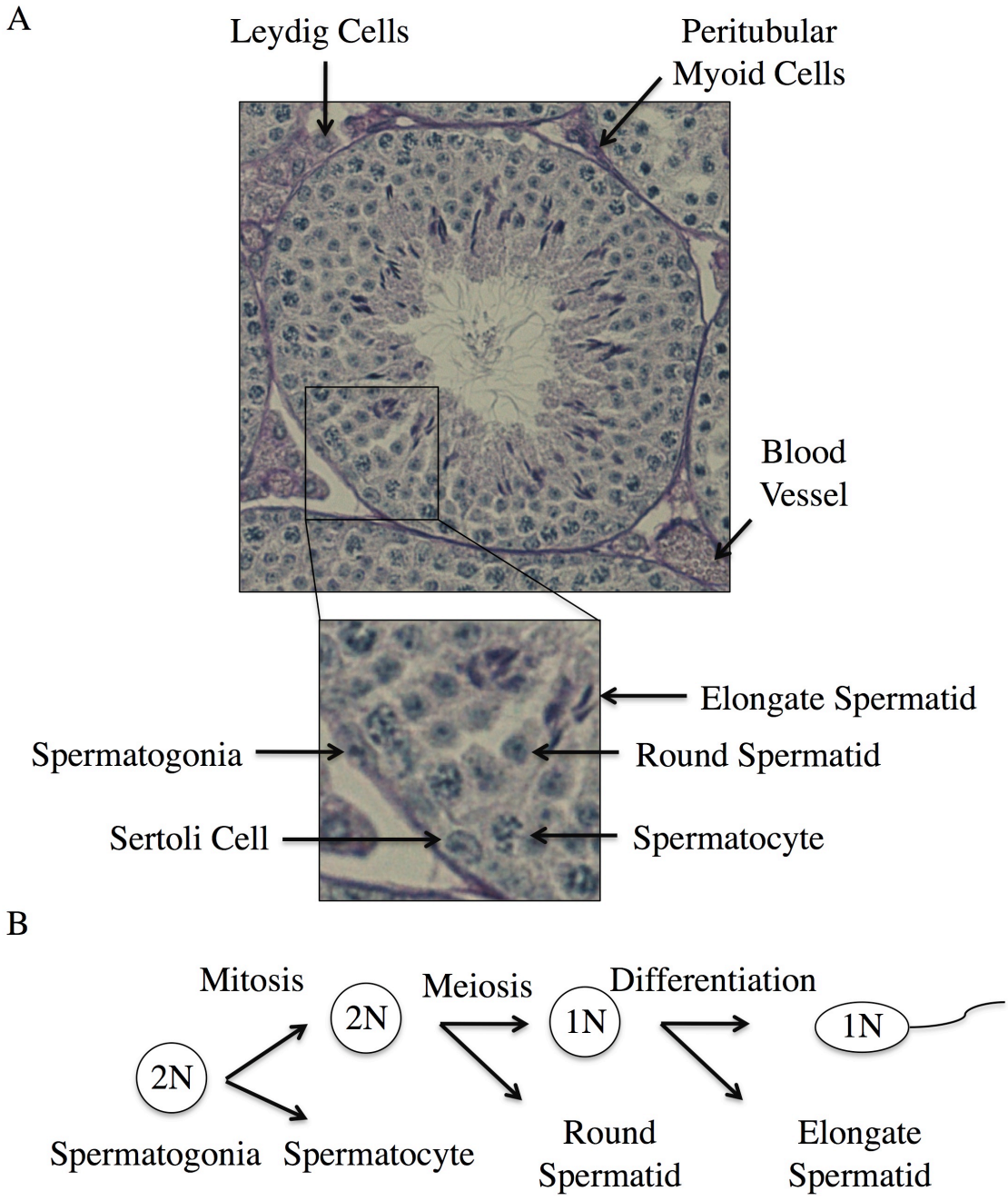

Figure 1.2 Testicular and seminiferous epithelium organization and spermatogenesis

A) Testicular cross section showing the organization of the different germ cell subtypes in the seminiferous epithelium and their B) division and differentiation steps.

become haploid germ cells, called spermatids (reviewed in[16]). Spermatids then undergo significant morphological and cellular changes in order to modify their structure from the early round cell shape to the elongated and distinct form of a sperm cell. Round spermatids differentiate to produce elongating spermatids, and upon maturation are released into the lumen of the tubule. The release of sperm from the seminiferous epithelium marks the end of one cycle of spermatogenesis, which in a mouse takes about 35 days to complete[17].

The specific timing and synchronization of the different divisions allows for the tubules to be divided in to specific stages, 12 in the mouse, based on the germ cell subtypes that are present[18, 19] (Figure 1.3A). This uniquely identifiable organization of the germ cell layers was originally identified in 1952 following what is now commonly known as PAS-H, or periodic acid-Schiff hematoxylin, staining of testicular cross sections[20]. This method takes advantage of the glycogen-rich acrosome, a cap-like structure that develops on the surface of the spermatid nuclei. The first step of the staining procedure utilizes periodic acid to oxidize the carbon bonds found in the glycogen molecules, generating two exposed aldehydes. The second chemical solution, Schiff's reagent, reacts with the aldehyde residues turning the acrosomes bright pink and hematoxylin is used to counterstain the nuclei purple. Using this technique, the development and movement of the acrosome was initially characterized, and the stages of spermatogenesis were further defined by the distinct germ cell layers present in each tubule[20]. One of the most easily recognizable of these stages in the mouse is stage XII, as it contains spermatocytes undergoing the first meiotic division, while other stages may be more identifiable by the progression of elongate spermatid development (Figure 1.3B).

1.1.3 Apoptosis in the testis

Apoptosis, or programmed cell death, is an important signaling pathway in the testis, both for the removal of potentially mutagenic damaged cells and for limiting the population of germ cells to match the supportive capacity of the Sertoli cells. Although there is much overlap, apoptosis is typically defined according to either of two major initiating signaling pathways: the intrinsic or the extrinsic signaling pathway.

The intrinsic, or mitochondrial-mediated apoptotic signaling pathway is usually initiated within the cell in response to various cellular stresses, such as DNA damage or heat shock (reviewed in[21]). In the testis, germ cells can become damaged through injury or irregular cell division, and under normal circumstances the cell is able to "sense" this through different self-check mechanisms. Once detected, a series of signals leads to the release of cytochrome c into the cytoplasm from the mitochondrial inner membrane space. Once in the cytosol, cytochrome c binds cysteine aspartate protease (caspase) 9 and apoptosis protease activating factor 1 (Apaf1) forming the apoptosome, initiating the cleavage of caspase 9[22]. This is an initiator caspase that, once activated, goes on to cleave and activate a caspase cascade that sends the cell into a terminal apoptotic pathway. There are also checkpoints along this pathway, including X-linked inhibitor of apoptosis (XIAP) that is able to bind and inhibit caspase 9[22].

The extrinsic, or death receptor-mediated apoptotic signaling pathway occurs when death ligands presented on one cell bind receptors on another cell, initiating the cell to undergo cell death. There are several different death ligands that belong to the tumor necrosis family (TNF) superfamily of ligands, including Fas ligand, TNF-related apoptosis inducing ligand (TRAIL), and TNFα, and each are capable of binding to their cognate receptor, Fas receptor (Fas), death receptor 4/5 (DR4/5), and TNFR. For apoptosis in the testis, it is generally accepted that Sertoli cells are the ligand-producing

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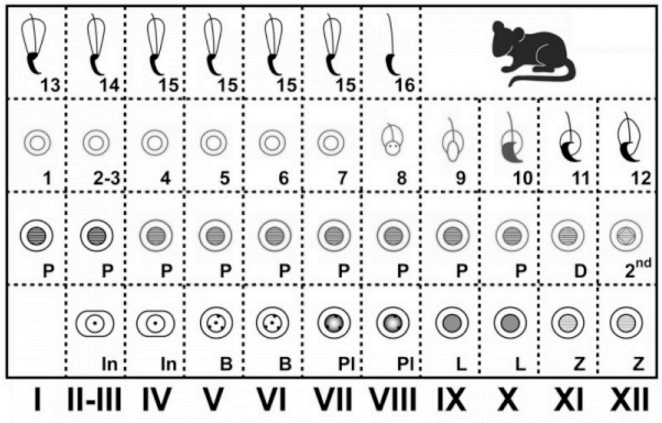

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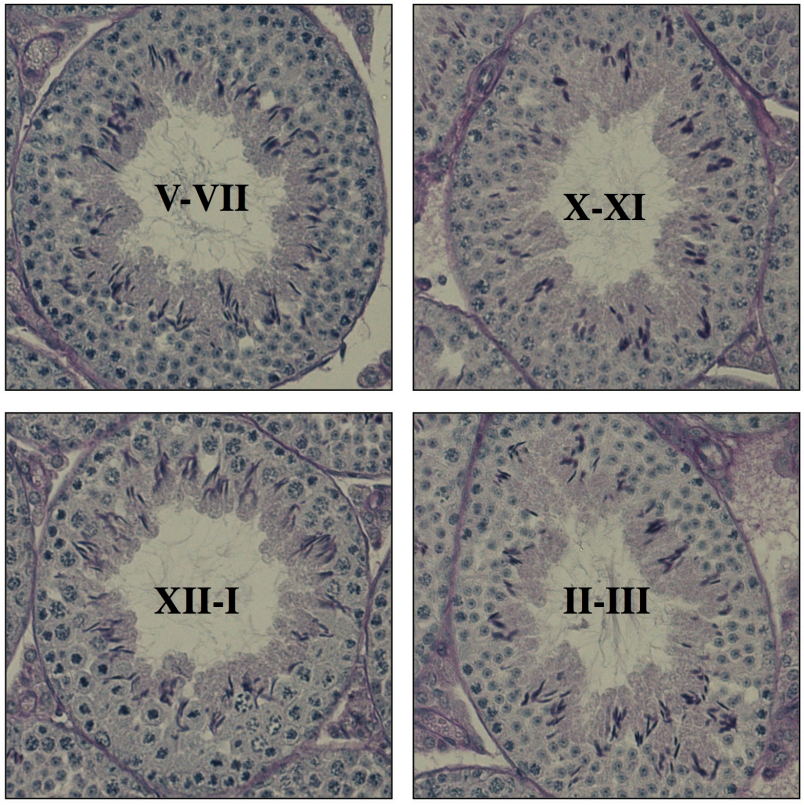

Figure 1.3 Tubule staging by germ cell subtype in the mouse

A) Chart representing the 12 stages of cycle of mouse seminiferous epithelium and B) histological images showing tubule cross-sections in various stages of the cycle.

cells, while the germ cells are the receptor-presenting cells[23-25] (Figure 1.4). Death ligands such as FasL are presented on the surface of Sertoli cells in groups of three, and bind to receptors like Fas on the surface of neighboring germ cells inducing clustering in similar groupings. Several accessory proteins are also recruited to the receptors, including the Fas-associated death domain (FADD) protein and a pair of caspase 8 proteins[26]. This entire complex is known as the death inducing signaling complex, or DISC. This close proximity of two caspase 8 molecules allows for their transactivation through a cleavage event, and the cleaved form of this initiator caspase then goes on to activate the downstream caspase cascade that ultimately results in apoptosis[27]. One important inhibitor of this pathway, cellular FLICE-like inhibitory protein (cFLIP), uses the proximity requirement of caspase 8 in order to block the death receptor mediated apoptosis[28]. This protein also contains a death domain similar to that of caspase 8, but it lacks the cleavage capability, so binding at the DISC therefore blocks a second caspase 8 molecule from binding, therefore blocking cleavage and apoptosis.

1.2 THE UBIQUITIN-PROTEASOME PATHWAY

1.2.1 Ubiquitin and the proteasome

Ubiquitin is a highly conserved, 8 kDa protein that is used to label proteins for a variety of purposes including cellular localization and degradation. It is attached to its target proteins through a three-step enzymatic process that covalently links it through the use of ATP (reviewed in[29]). First, the ubiquitin activated by an E1 ubiquitin-activating enzyme that hydrolyzes ATP to ADP to first activate the ubiquitin molecule then to covalently attach the molecule to itself (Figure 1.5A). Secondly, an E2 ubiquitin conjugating enzyme facilitates the transfer of the ubiquitin molecule from the E1 to itself. Lastly, an E3 ubiquitin ligase targets the substrate protein and transfers the ubiquitin

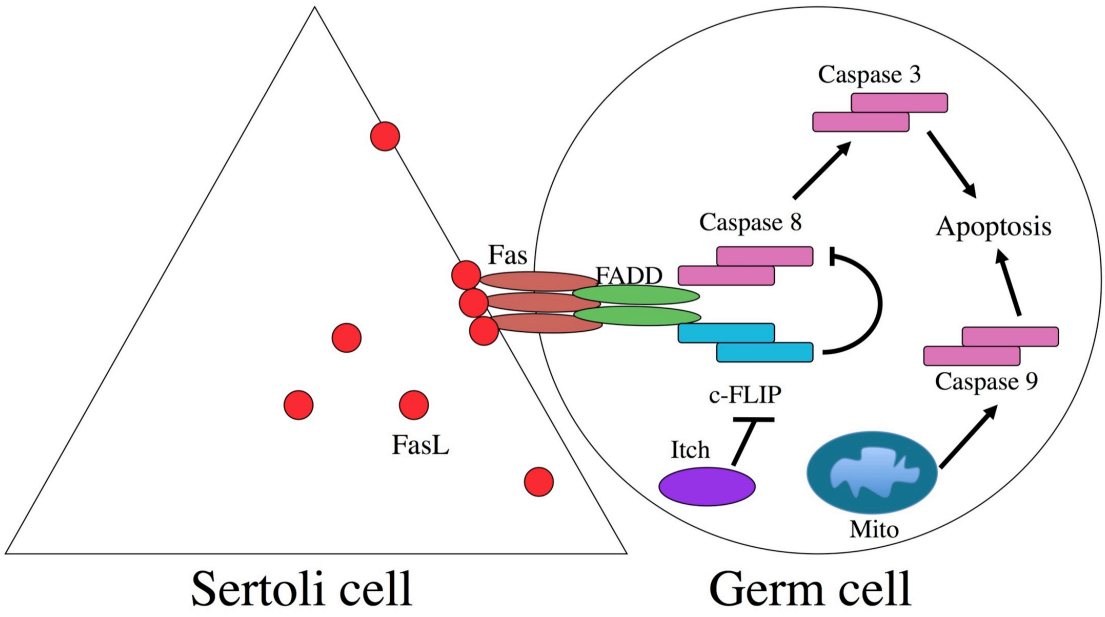

Figure 1.4 Extrinsic and intrinsic apoptosis signaling in the testis

In the testis, Sertoli cell-mediated extrinsic signaling is initiated by the production of FasL and its binding to Fas on neighboring germ cells. Germ cell-mediated intrinsic signaling is driven by the release of cytochrome c from the mitochondria.

molecule from the E2 and covalently links it to its final target. The protein substrate can then either be released for its specific purpose or continue through this process multiple times to add on a chain of ubiquitin molecules on to the first ubiquitin. For proteins that are tagged with multiple ubiquitin proteins, it is thought that the initial ubiquitin molecule is the signal to add on the additional ubiquitin chain.

The number of ubiquitin molecules and the specific amino acid residues on the target protein at which they are bound play a role in the fate of the protein. Target proteins can be mono-ubiquitinated or poly-ubiquititinated using one of the seven available lysine residues on the ubiquitin molecule[30]. The most common linkages involve lysine 48 and lysine 63, although other less common linkages do occur. These various linkages can signal the protein for different cellular locations, such as the plasma membrane or lysosomes, or target them for degradation by the 26S proteasome. For example, a poly-ubiquitinated chain using lysine 63 usually signals a target protein for a specific localization, while a poly-ubiquitinated chain using lysine 63 is used as means of protein expression control and sends the targeted protein to the 26S proteasome for degradation. The proteasome is a cylinder-shaped protein complex made up of several subunits. It functions to breakdown unfolded, mis-folded, and unneeded proteins in the cytoplasm by breaking the peptide bonds between amino acids.

1.2.2 E3 Ubiquitin Ligases and Itch

The nature of the ubiquitin pathway gives substrate specificity to the final enzyme in the pathway, the E3 ubiquitin ligase, and therefore while there are only a few E1s and several E2s, there are hundreds of known and possibly more yet to be identified E3 ligases. There are two major classes of E3 ligases, homologous to E6-AP carboxy terminus (HECT) and really interesting new gene (RING) family members, and they

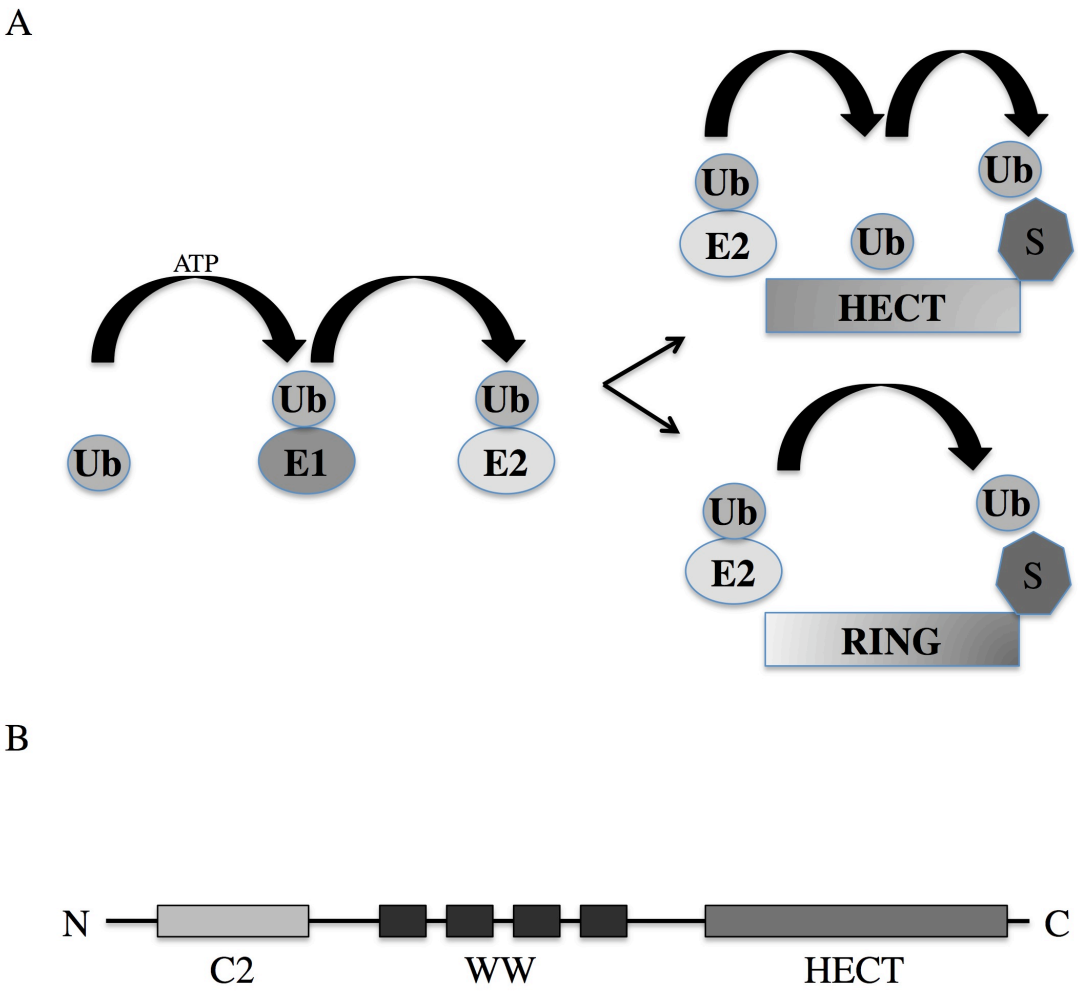

Figure 1.5 HECT and RING E3 ubiquitin ligases and the protein structure of Itch

A) HECT E3 ligases act as ubiquitin intermediates while RING E3 ligases act as scaffolding proteins, bringing the E2 and substrate into close contact. B) Itch consists of a N-terminal C2 lipid binding domain, four WW protein-binding domains, and a C-terminal enzymatically active HECT domain.

differ in their sequence and mechanism by which they transfer the ubiquitin molecule (reviewed in[31]). RING E3 ligases act as scaffold proteins, bringing the E2 conjugating enzyme and target protein in close proximity and transferring the ubiquitin from one to the other (Figure 1.5A). On the other hand, HECT E3 ligases act as intermediates, first transferring the ubiquitin molecule from the E2 to itself, and then transferring it onto the target protein. This allows for the detection of an E3-ubiquitin intermediate that is not observed with the RING E3s.

One E3 ubiquitin ligase, Itch, belongs to the HECT family of E3s, and has a molecular weight of 113kDa (reviewed in[32]). Studies have shown that Itch is expressed throughout the tissues and organs, and that this expression appears to be consistent throughout development. It consists of a N-terminal lipid binding C2 domain, four internal WW protein-binding domains, and a C-terminal enzymatically-active HECT domain (Figure 1.5B). The WW protein interacting domains are so called for the two conserved tryptophan residues that bind proline rich sequences in target proteins, specifically PPLP or PPXY sequences (reviewed in[29]).

1.2.3 The proteasome pathway in the testis

Recent evidence has demonstrated the importance of the ubiquitin-proteasome pathway in the testis, both in the developing haploid germ cells where cellular remodeling and chromatin condensing is occurring and in the supportive Sertoli cells which must remove the residual waste that is left behind (reviewed in[33]). Whole body tissue analysis in the rat revealed that the testis had the highest rate of ubiquitination among all of the organs examined[34], suggesting that this pathway may play an important regulatory role. In fact, several testis-specific ubiquitin related proteins have already been identified, and the loss of some of these has been shown to cause significant testicular

effects. CUL4A, an E3 ligase important in DNA replication and the cell cycle, is required for spermatogenesis, and although viable, male *Cul4A*-/- mice are completely infertile and fail to develop later stage germ cells[35]. Knockout mice lacking the deubiquitinating enzyme USP2 appeared morphologically normal, but the males were only able to produce 12% as many offspring compared to wild type due to a defect in their sperm's ability to fertilize the egg[36]. Some phenotypes, however, are not as severe, such as the testis specific E2 ubiquitin conjugating enzyme UBC4, where there knockout mouse strain shows a delay in testis development but otherwise normal spermatogenesis[37].

Many of the testicular targets of the ubiquitin-proteasome pathway that have been described have been in the germ cells due to their extensive cellular remodeling, including the removal and degradation of the histones to allow for the condensing of the DNA[38]. However the Sertoli cells also utilize this system in order to regulate protein expression and clean up the residual waste that is left behind from the released spermatids. One protein that was found to be regulated by this pathway is the tight junction protein occludin, an important mediator in the blood testis barrier (BTB), and interestingly the E3 ligase identified as being responsible for targeting its ubiquitination was Itch[39]. As the germ cells differentiate and move from the basal compartment to the adluminal compartment, they must cross the specialized yet otherwise impermeable contact points formed by neighboring Sertoli cells[40]. These junction sites are therefore in constant motion, with the proteins disassembling and reorganizing after the germ cells have passed. Itch was shown to aid in this recycling of the junctional proteins by targeting occludin for proteasomal degradation, with the protein levels of Itch being inversely correlated to those of occludin[39]. It was also shown that this regulation could be inhibited by the proteasome inhibitor MG-132, and in fact caused an increase in Itch-occludin co-immunoprecipitation products, indicating that the mechanism by which Itch

acts to regulate the levels of occludin is by targeting it for degradation by the 26S proteasome.

1.3 *ITCHY* MICE AND THE IMMUNE SYSTEM

1.3.1 Generation of the *itchy* mice and identification of Itch

Much of the progress in deciphering the role of the E3 ligase Itch during development has stemmed from the generation and identification of Itch loss-of-function mutant mice. In 1994 in an initial study of the coat color gene *agouti*, ionizing radiation was used to generate a series of recessive mutants, and unexpectedly one of these mutants, *nonagouti-lethal 18H* (a^{18H}), also presented with a severe immunological phenotype[41]. Since none of the other brown *agouti* mutants showed similar symptoms, it was determined unlikely that the altered coat color gene was the source of the immune disorder, so the researchers used genomic mapping and fragment analysis to identify the mutation site. It was found that the ionizing radiation had resulted in a chromosomal inversion that included the upstream region of the *agouti* gene and the downstream portion of a yet unidentified gene[41] (Figure 1.6A). Even on a C57 black background, disruption of the *agouti* gene caused these animals to display a brown agouti coat color (Figure 1.6B).

This same group later used restriction enzymes and sequence analysis to establish that the a^{18H} mouse genome contained a single simple inversion with two short deleted sequences at each of the breaks[42]. From the cDNA and the predicted protein sequence, the protein was identified as an E3 ubiquitin ligase, endearingly named Itch by the group for the dermatitis and resultant scratching phenotype that the mice presented with. From the predicted sequence they were able to determine that this protein contained a C-terminal HECT domain and four protein-binding WW domains, although later it was also

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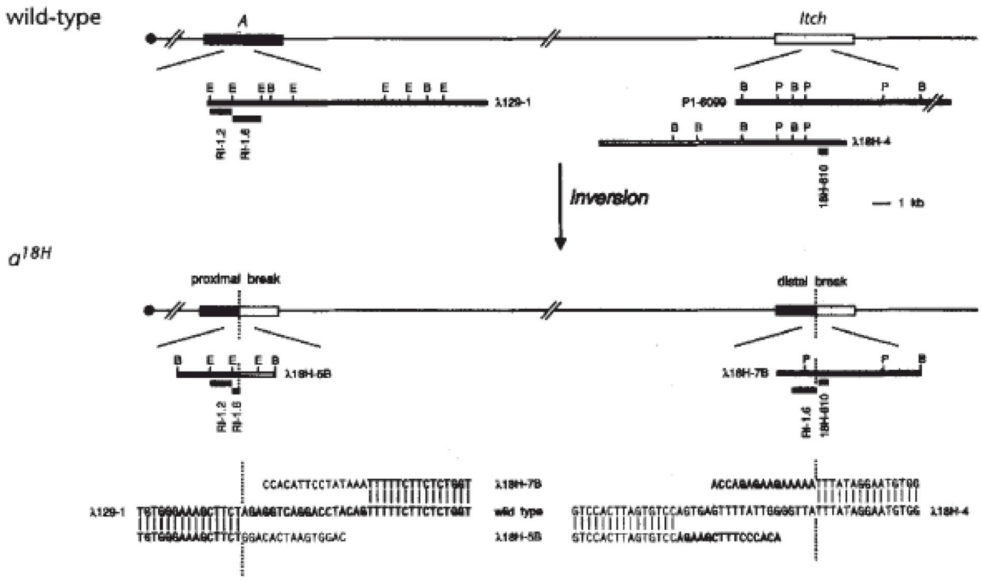

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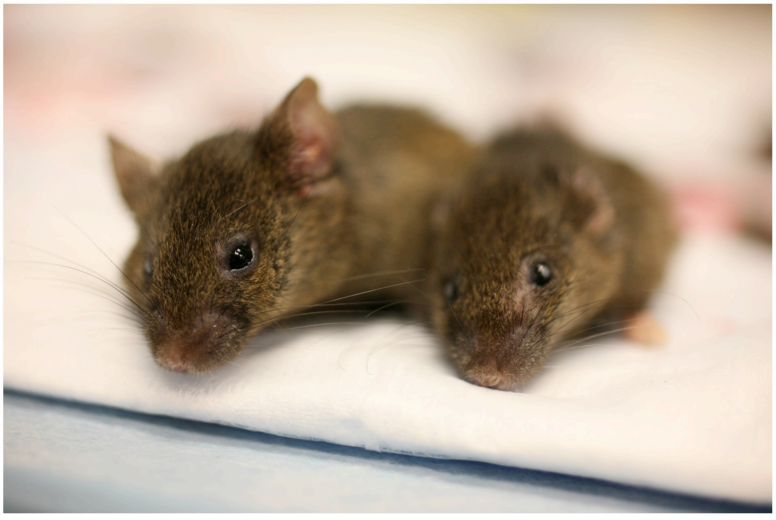


Figure 1.6 Chromosomal inversion resulting in the generation of the *itchy* mice

A) Ionizing radiation randomly generated a chromosomal inversion, disrupting the coat color gene *agouti* and the neighboring gene that was later identified as *itch*. B) The decreased *agouti* makes these mice appear brown even on a C57BL/6 background.

found to have an N-terminus C2 membrane-binding domain[29]. Northern blot analysis confirmed that this was indeed the RNA that was missing from the *itchy* mice, and tissue expression analysis revealed that the Itch RNA was expressed in all of the tissues examined[42].

1.3.2 The role of Itch in the immune system

Generation of the *itchy* mice led to a wealth of knowledge about the role that Itch plays in the developing and adult immune system (reviewed in[29]). Autoimmune diseases, like the one that the *itchy* mice develop, describe a collection of conditions where ultimately the body is no longer able to distinguish between self and non-self. This most typically occurs due to a malfunction of T cells, which are responsible for targeting and destroying non-self infections[43]. Under normal conditions, the T cells are trained early on to recognize what is 'self', and therefore do not mount an attack against the body's own cells. Unfortunately this ability is sometimes lost, and the immune system begins to target the 'self' antigens as if they were invading pathogens. This is what occurs in the *itchy* mice, an overproduction of immune cells that quickly invade and deposit into the surrounding tissues and organs[44]. As early as 2 months, these mice are already producing immunoglobulins that recognize self-antigens and deposit in the animal's tissues. At 5 months they develop dermatitis, which leads to extensive scratching of the head and neck, and severe lymphoproliferation and internal inflammation. These animals typically die between 6 and 9 months of age, usually from asphyxia due to pulmonary inflammation and invasion of immune cells, and interestingly their survival rates can be completely recovered if crossed with a *Rag1*-/- mouse that lacks mature lymphocytes, indicating that death of the *itchy* mice results solely from the infiltration of the immune system[29].

The work done with the *itchy* mice has led to the identification of a number of immune-related protein targets, while a limited number of studies outside of the immune system have pointed to a few more diverse substrates. JunB, a transcription factor important in the differentiation of T_h1 and T_h2 cells, is targeted for degradation by Itch, and its subsequent increase in the *itchy* mice leads to an over-proliferation of Th2 cells and IgG1 levels[43]. Another transcription factor c-Jun is also increased in the *itchy* mice, and the lack of Itch-mediated protein degradation leads to an increase in T cell activation[43]. Examination of the surface markers of these T cells revealed that although it is not required for the initial development of these cells, the lack of Itch later in adulthood results in constitutively active T cells. One of the more significant roles Itch has been identified to play in the immune system is its regulation of Notch1, a transmembrane protein that gets cleaved after ligand binding, and over-expression of the cellular fragment leads to a phenotype similar to that of the *itchy* mice[29]. Itch has also been found to regulate several other important pathways, and its ubiquitous expression suggests that it may play multiple tissue and/or cell specific roles. The anti-apoptotic protein cFLIP, which blocks activation of caspase 8 at the DISC, gets ubiquitinated by Itch and targeted for degradation, leading to the removal of this inhibition and therefore promoting apoptosis[45]. Itch is also expressed by the Sertoli cells in the testis where it regulates the tight junction protein occludin, an important mediator of the blood testis barrier[39, 46].

1.3.3 The immune system in the testis

The testis has long been thought of as an "immune privileged" organ due to the fact that the developing germ cells are protected from the immune system even though germ cells first develop after the so-called "self-recognition" period. Mature spermatids, which express numerous sperm-specific surface proteins, do not appear until

spermatogenesis completes its first cycle at the onset of puberty, years after immune tolerance is established[47]. This isn't from a lack of presence in the reproductive tract, as immune cells can be found throughout the epididymis and within the interstitial space of the testis, so other physical barriers must be in place to protect the germ cells from direct attack or inflammatory effects (reviewed in[48]). The term "immune privilege" can be defined as the specific characteristic of an organ or tissue to either 1) be transplanted to another region of the body without causing an immune response or 2) accept tissue or cells from another source without inducing an immune-directed rejection[13]. The testis is unique in that it possesses both of these characteristics, although the exact mechanisms remain mostly unclear[49].

Several features of the testis have been identified that are thought to provide this organ with protection against the circulating immune system (reviewed in[13]). What was originally thought to be the sole contributor to the testis' immune privilege is the blood-testis barrier, a physical structure created by junctional proteins between neighboring Sertoli cells. This structure separates the seminiferous epithelium into an adluminal and basal compartment, while at the same time preventing the circulating immune system access to the cells most susceptible to an immune attack[47]. This physical barrier can not fully explain the observed protection, however, as grafts placed within the interstitium are still afforded protection against invading immune cells[13]. Another feature of Sertoli cells that is thought to assist in this protection is their high surface expression of Fas ligand[47]. It is thought that Sertoli cells are able to specifically target T cells for apoptosis due to their expression of Fas receptor, although there is some dispute as other FasL expressing cells such as in the pancreas do not show this same ability[47]. It may also be a difference in the immune cell population in the testis that allows for the protection of the germ cells. The most common immune cells found in the testis are macrophages, specifically

resident macrophages, and studies have shown that these specific cells have unique cytokine expression patterns that minimize and/or even inhibit the immune response[50, 51].

1.4 Dissertation Aims

This project initially set out to examine the role of the negative regulator Itch on the anti-apoptotic protein cFLIP and germ cell apoptosis following peri-pubertal exposure to mono-(2-ethylhexyl) phthalate (MEHP). Although a protection was hypothesized, dosing experiments using PND 28 C57BL/6J and *itchy* mice revealed similar germ cell apoptotic indexes between the genotypes, and western blot analysis revealed no significant alterations in cFLIP levels in the *itchy* mice. During this toxicant study, however, several consistent and identifiable differences were noted between the *itchy* and wild type mice, and from there a developmental analysis was initiated. Utilizing the knockout mouse model *itchy*, this work examines the role of the E3 ligase Itch in reproduction and male testicular development.

As an initial analysis, the physical and reproductive characteristics of the *itchy* mice were evaluated (**Chapter 3**). Body and testis weights, spermatid head counts, litter sizes and embryo implantation numbers were used to get a gross overview of the reproductive health of the *itchy* mice and the role of Itch during fetal and gamete development. Next, a detailed histological analysis was conducted on the testes of the *itchy* mice at two time points, peri-pubertal PND 28 and adult PND 56, and compared to age-matched wild type mice (**Chapter 4**). These ages were chosen due to their developmental significance, with PND 28 catching the end of the first wave of germ cell apoptosis and PND 56 occurring after a full round of spermatogenesis. Although no differences were observed in apoptosis following toxicant treatment, the *itchy* mice had basal levels that were higher than those of their C57BL/6J counterparts, so the apoptotic

signaling pathways were analyzed (**Chapter 5**). Finally, the role of Itch in the testis was examined by evaluating several previously identified protein substrates (**Chapter 6**). However, when this failed to turn up any significant differences, the influence of the over active immune system and the infiltrating immune cells were put into question. Although there were a number of repeatable and detrimental testicular and reproductive phenotypes in the *itchy* mice, the role of Itch in the testis remains mostly unanswered, and future work is needed to decipher these questions (**Chapter 7**).

Chapter 2: Materials and Methods

2.1 ANIMALS:

All mice used in the experiments described were housed in the Animal Resource Center at The University of Texas at Austin. The mouse room was kept at a constant temperature of 22 ± 0.5°C at 35-70% humidity with a 12L:12D photoperiod. To enhance breeding, mice were fed a high-energy diet containing 9% fat (5P06 Prolab RMH 2000) and water *ad libitum*. All experiments using mice were preformed in accordance with the guidelines of The University of Texas at Austin's Institutional Animal Care and Use Committee in compliance with guidelines established by the National Institute of Health. Mating pairs of wild type C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in house. *Itchy* mice (Itch-/-) on a C57BL/6J background were a generous gift from Dr. Lydia Matesic at the University of South Carolina, Columbia, South Carolina[52]. These *nonagouti-lethal 18H* (a^{18H}) mice were originally generated through a radiation-induced chromosomal inversion[41] and further characterized as having a disrupted gene that was later termed Itch[42]. Heterozygous breeding pairs were initially set up, and from the F1 generation offspring, Itch+/+ (wild type C57BL/6J) and Itch-/- (*itchy*) breeding colonies were established. Cross breeding experiments were performed using *itchy* mice from the breeding colonies and wild type C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME).

2.2 GENOTYPING PCR AND PRIMERS

Wild type C57BL/6J and *itchy* mice were confirmed using genotyping PCR with primers specific for the Itch gene (protocol from Dr. Lydia Matesic). Tail clippings were collected and digested overnight with Proteinase K. Total genomic DNA was ethanol precipitated and PCR was performed using Taq polymerase. The primers used included

individual wild type (5' – atc gtc tac tca ccc cac ata agg – 3') and mutant (5' – aag aag cag cag aga caa cga gtg – 3') forward primers that share a common reverse primer (5' – tct atg ctc tgt tgt ctc cca tgc – 3'). The wild type primer and common primer results in a 194 bp band, while the mutant primer mixed with the common primer results in a 294 bp band.

2.3 PHYSIOLOGICAL AND REPRODUCTIVE CHARACTERIZATION

Body and testis weights were recorded at PND 28 and PND 56. These ages were selected in order to evaluate two important testicular developmental ages, peri-pubertal (PND 28) and adult (PND 56). The testis weights were expressed as an average of the right and left testis weights, and the testis/body weight ratio as an average testis weight in grams divided by the body weight in kilograms. Spleen weights were also measured at PND 28 and PND 56, and represented as a ratio to body weight. A minimum number of five mice were used for each genotype in each age group. Litter sizes were determined by counting the total number of pups at PND 0 (pups born) and by counting the total number of pups that survived until weaning at PND 21 (pups survived). Litters that were born but either eaten or not taken care of by the mother were counted as zero pups survived. A minimum number of 12 females/mating cages were used for each wild type and *itchy* group. Each female was left to breed until they ceased having pups for 2 months or died. For cross-mating experiments, a minimum of 4 females/mating cages and a minimum of 11 litters were counted for each of the two crosses.

2.4 SPERMATID HEAD COUNTS

Testes were collected from PND 56 mice and flash frozen in liquid N_2. Frozen testes were gently homogenized in a solution containing 0.9% w/v NaCl and 10% v/v dimethyl sulfoxide (DMSO). Homogenization-resistant spermatid heads were counted on

a standard hemocytometer using a Nikon E800 microscope. The average number of spermatid heads for each genotype was determined using a single testis from 8 individual mice, and each testis sample was counted 3 times.

2.5 UTERI AND IMPLANTATION SITE EXAMINATIONS

Pregnant female mice were monitored throughout their pregnancy and allowed to have a normal and undisturbed birth. For post-uteri examinations, females were sacrificed the day following birth (PND 1) by CO_2 asphyxiation, and following pictorial documentation, the uterine horns along with any contents were completely removed. If the uteri appeared to contain mal-developed and/or resorbed pups, then each of the distal ends were cut allowing for the remnants to be pushed out. None of the fetuses found still in the uteri responded to stimuli, and pictures were taken of all that were found. The number of implantation sites was determined using a 10% ammonium sulfide solution. Virgin females were mated with adult males and allowed have a normal and undisturbed birth. Five days after birth, the numbers of pups were counted and females were sacrificed by CO_2 asphyxiation. The uterine horns were removed and completely submersed in a 10% ammonium sulfide solution (Fisher, A705-225) for 1 hour. Sites of fetal implantation, which appear as a black spot, were counted and compared to the number of pups born.

2.6 TISSUE COLLECTION AND PROTEIN EXTRACTION

Whole testes and other tissues were collected and immediately flash frozen in liquid N_2, then stored at -80°C until needed for protein extraction. Isolated tubules were dissected out of freshly collected whole testes immediately after sacrifice. Both testes were detunicated into a 0.1% collagenase (Sigma, C2674) solution in HBSS (GIBCO, 14170-112), and put in a shaking water bath at 34°C for 15 minutes. Isolated tubules

were removed from solution, washed in PBS, and gently spun down to remove the excess liquid. Total protein was collected from frozen whole testes and fresh isolated tubules by homogenizing in Radio-Immunoprecipitation Assay (RIPA) buffer with protease inhibitors. The concentration of each sample was determined using the Biorad DC Protein Assay Lowery Method.

2.7 WESTERN BLOT ANALYSIS

For each sample, 30μg was separated using a 4-12% NuPAGE gradient gel, transferred to a PVDF membrane, and blocked using a 5% milk solution. Quantification was determined using antibodies specific for Itch (BD Trans, 611199, 1:1000), FasL (Santa Cruz, sc834, 1:1000), TRAIL (Zymed, 40-3900, 1:1000), cFLIP (Dave-2, Alexis, ALX-804-127, 1:1000), c-Jun (Cell Signaling, 9165, 1:1000), occludin (Abcam, ab31721, 1:1000), Fas (Upstate, 05-351, 1:1000), DR5 (Novus, NB600-1091, 1:1000), pERK (Cell Signaling, 4370, 1:1000), ERK (Cell Signaling, 9102, 1:1000), LHR (Santa Cruz, 25828, 1:1000), cyclin D (Cell Signaling, 2926, 1:1000), Akt (Cell Signaling, 9272, 1:1000), pAkt Ser473 (Cell Signaling, 4060, 1:1000), and $p27^{kip1}$ (Cell Signaling, 3698, :1000), along with the respective secondary antibodies. For detection of IgG levels, anti-mouse and anti-rat secondary antibodies from Cell Signaling (7076 and 7077) and Santa Cruz (2031 and 2032) were used in the absence of primary antibodies. Actin (Santa Cruz, sc1616, 1:1000) was used as a loading control. Quantification was performed using the ImageJ software (NIH).

2.8 TESTICULAR HISTOLOGY

Whole testes were collected and fixed in Bouin's solution, then washed in 70% ethanol saturated in $LiCO_3$. Paraffin-embedded testicular cross sections (5 μm) were examined using periodic acid-Schiff-hematoxylin (PAS-H) staining. Sections were

viewed on Nikon E800 microscope and images were captured using a Nikon Digital DS camera and NIS Elements software.

2.9 MEIOTIC QUANTIFICATION

The percentage of essentially round seminiferous tubules containing meiotic figures was quantified as a percentage of the total number of tubules. At least 2 sections were counted from each animal, and at least 6 animals were counted in each age group.

2.10 TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE-MEDIATED DIGOXIGENIN-DEOXYURIDINE TRIPHOSPHATE NICK END LABELING

The germ cell apoptotic index was determined through terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine triphosphate nick end labeling (TUNEL) analysis using an Apoptag™ kit (Chemicon, S7100). Paraffin-embedded testicular cross sections (5 μm) were enzymatically labeled and the slides were imaged using a Nikon E800 microscope. The apoptotic index was determined by calculating the percentage of essentially round tubules that contained more than three TUNEL-positive germ cells. At least 2 sections were counted from each animal, and at least 8 animals were counted in each age group.

2.11 TESTICULAR IMMUNOHISTOCHEMISTRY

Paraffin-embedded testicular cross sections (5 μm) were deparaffinized, rehydrated, and antigens were unmasked by boiling in sodium citrate. Hydrogen peroxide was used to inhibit endogenous peroxidases, while horse serum (Vector laboratories, S-2000) was used to block nonspecific antibody binding. The antibody was diluted in blocking serum and slides were incubated overnight at 4°C. The primary antibody was detected using a biotinylated anti-rabbit secondary, Vectastain ABC reagent (Vector Laboratories, PK-6101), and DAB peroxidase substrate (Vector laboratories, SK-4100).

Sox9 (Millipore, AB5535, 1:500) was used as a marker for Sertoli cells, and the average number per tubule was determined by averaging the number of positive signals in 50 essentially round tubules. Cleaved caspase 9 (Cell Signaling, 9509, 1:100) was used as an indicator of intrinsic apoptosis, and the index was determined by calculating the percentage of essentially round tubules that contained more than three caspase 9-positive germ cells. At least 3 sections were counted from each animal, and at least 2 animals were counted in each age group. For histology, FoxO1 (2880, Cell Signaling, 1:100) was used to label early spermatogonia.

2.12 MICROARRAY ANALYSIS

Whole testes were collected from PND 28 and PND 56 C57BL/6J and *itchy* mice and immediately immersed in RNAlater (Sigma, R0901). Samples were shipped on dry ice to Phalanx Biotech Group, Belmont, CA, where RNA isolation and OneArray® total gene expression profiling was performed. Results were given for all of the genes examined, and the *itchy* samples with a reported value measuring 4-fold different than control ($\leq \log_2 2 \leq$) were examined further.

2.13 TWO-DIMENSIONAL (2D) GEL ELECTROPHORESIS

Whole testes were collected from PND 28 and PND 56 C57BL/6J and *itchy* mice and flash frozen liquid N_2, then shipped on dry ice to Applied Biomics, Hayword, CA. Wild type C57BL/6J proteins were labeled with Cy3 (green) while *itchy* samples were labeled with Cy5 (red). On an overlay gel, proteins that are found in similar concentrations in the two genotypes appear yellow, while proteins that are higher in either genotype appear in that protein's color. Spots were selected based on availability and the size of the difference from control, and identified using MALDI-MS/MS (Applied Biomics, CA).

2.14 STATISTICAL ANALYSIS

Statistical results are expressed as the mean ± SEM. The data were analyzed using a Student's t-test or a one-way ANOVA (JMP 9) and were considered statistically significant when $p < 0.05$.

Chapter 3: Physiological and Reproductive Characterization of *Itchy* Mice During Early Development

3.1 Introduction and Rationale

Spermatogenesis is the intricate process in which immature stem germ cells divide and differentiate under the care and supervision of somatic Sertoli cells to become mature and functional sperm. This process relies strongly on the rapid turnover of proteins, both in the developing germ cells, which must quickly change their cellular composition as they differentiate into later stages, and in the supporting Sertoli cells, which must collect and degrade the residual cytoplasm left over by the released spermatids (reviewed in[33]). Recent evidence has pointed to the importance of the ubiquitin-proteasome pathway in this protein turnover, and several testis-specific proteins have been identified[37, 38]. The ubiquitin pathway is a three-step enzymatic process that covalently links the 8kDa ubiquitin molecule to specific target proteins, which then acts as a signal to direct the substrate for cellular localization or degradation by the 26S proteasome (reviewed in[29]). This pathway is initiated by an E1 ubiquitin activating enzyme, which utilizes ATP to activate the ubiquitin molecule, followed by an E2 ubiquitin conjugating enzyme, which transfers the ubiquitin to the final E3 ubiquitin ligase, which covalently attaches the ubiquitin to the target protein. Due to the nature of this pathway, it is the E3 ligase that provides substrate specificity, and where there are only a few E1s, several hundred E3s have been identified.

One such E3 ligase, Itch, has been well studied for its role in the immune system, but was first identified in a random mutation screen of a set mice deficient in the coat color gene *agouti*[41]. After a dose of ionizing radiation, one set of mice were found to have a late onset autoimmune disease, presenting with severe dermatitis which lead to itching

and scratching, so they were endearingly named *itchy* mice. Later the disrupted gene was identified to be an E3 ubiquitin ligase, and in keeping with this knockout strain the protein was termed Itch[42]. Much of the progress on deciphering the role of Itch has come from studies using the *itchy* mice and its importance in the developing immune system, but the variety of identified targets and vast tissue expression suggests that Itch may play a series of diverse and possibly cell-specific functions (reviewed in[29]). Although links have been made between Itch and apoptosis, and the ubiquitin system is known be important during spermatogenesis, the role of Itch during testis development and reproduction remains unknown.

This chapter investigates the reproductive and developmental phenotype of the *itchy* mice by examining their gross physical characteristics and breeding capabilities compared to wild type C57BL/6J mice. Along with whole body characteristics, specific testicular measurements were used to determine reproductive function, such as spermatid head counts for germ cell production and Sox9 staining for Sertoli cell development. Mating experiments were also used to determine fertility and fecundity of the *itchy* mice, both as mating pairs and as individual breeding sexes with wild type mice. This study identified alterations in several gross and specific testicular characteristics throughout development, while some of the measured functions remained wholly unchanged. Breeding experiments revealed a possible role for Itch during embryonic development and maternal-fetal support. Together these results suggest that although the *itchy* mice are viable and fertile, this particular E3 ligase plays an important and functional significant role during reproductive development.

3.2 RESULTS

3.2.1 *Itchy* mice have smaller body weights and testis weights compared to wild type mice

For the initial analysis of *itchy* development, body weights and testis weights were recorded at two developmentally important time points, after the first wave of apoptosis at PND 28 and at the adult age PND 56. At PND 28, *itchy* mice had significantly smaller body weights than C57BL/6J mice (*itchy* 13.72±0.31g and C57 15.43±0.46g) and this trend continues throughout adulthood (Figure 3.1A). The testis weights were not significantly decreased at PND 56 (*itchy* 0.088±0.002g and C57 0.098±0.001g), indicating that this difference in testis weight occurs after the first wave of spermatogenesis (Figure 3.1B). A comparison of the testis to body weight ratios revealed that *itchy* mice consistently have higher ratios than their wild type counterparts, both at PND 28 (*itchy* 3.30±0.09g/kg and C57 2.96±0.05g/kg) and at PND 56 (*itchy* 4.25±0.08g/kg and C57 3.91±0.07g/kg), which results from the body weights being more significantly impacted than the testis weights (Figure 3.1C).

3.2.2 *Itchy* mice have fewer pups per litter than C57BL/6J mice, but their survival rates are not significantly different.

The reproductive fecundity and survival rate of the *itchy* mice was examined by comparing the average number of pups born per litter (total pups at PND 0) and the number of pups that were still alive at the date of wean (number survived). For C57BL/6J mice, 63 litters from 17 breeding females were counted, giving an average of 3.7 litters per female. For *itchy* mice, 65 litters from 26 breeding females were counted, giving a lower average of 2.5 litters per female. This difference can be accounted for by the immunological phenotype of the *itchy* mothers, which were either too sick or did not live long enough for same number of litters. The number of *itchy* pups born per litter was

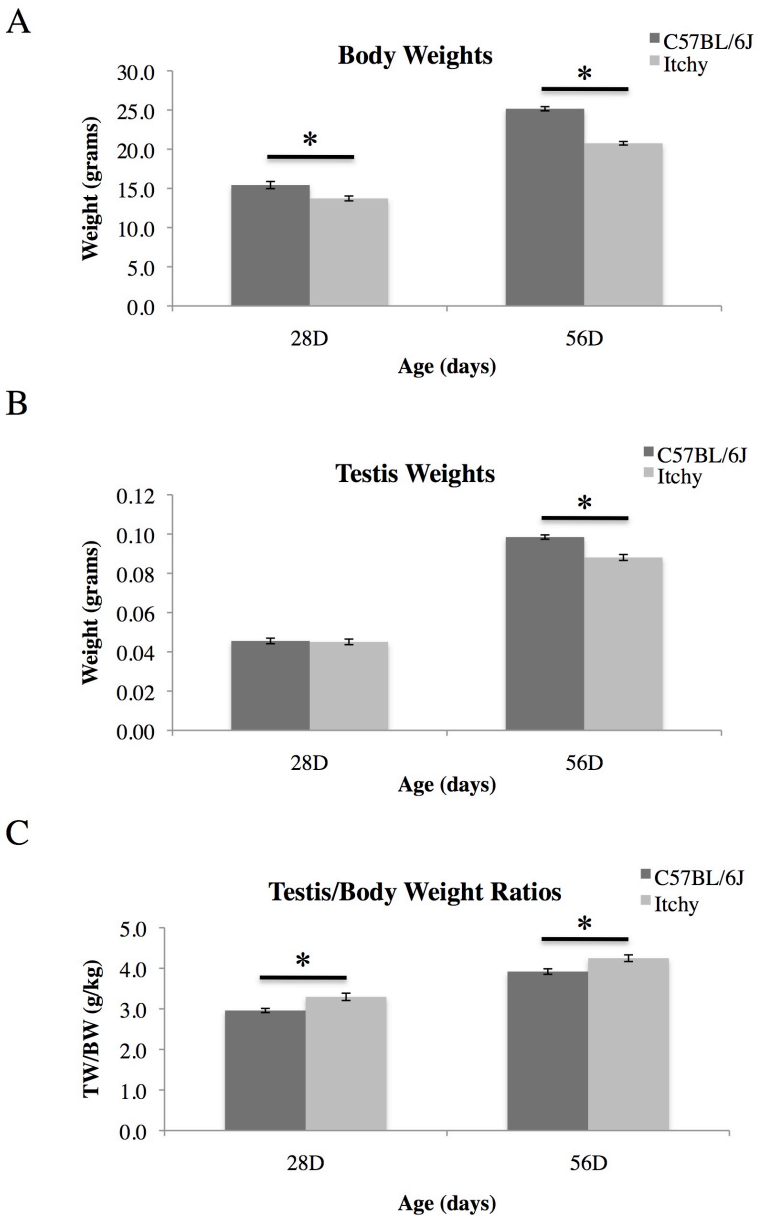


Figure 3.1 Physical and testicular characteristics of C57BL/6J and *itchy* mice.

A) Body and B) testis weights (g), and C) testis/body weight ratios (kg/g) of C57BL/6J and *itchy* mice at PND 28 and PND 56. *Itchy* mice are overall smaller than their wild type counterparts. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

significantly decreased compared to C57BL/6J (C57 7.84±0.30 and *itchy* 5.28±0.23), suggesting that there may be a reproductive role for Itch during conception and/or pregnancy (Figure 3.2A). Interestingly, the average number of *itchy* mice that survived was not statistically different from wild type (C57 3.76±0.37 and *itchy* 3.51±0.24). Further examination revealed that a larger number of entire C57BL/6J litters did not survive, due to either being eaten or not being cared for by the mother (Figure 3.2B). Including the litters where more than half of the pups did not survive increased this gap further.

3.2.3 The total number of spermatid heads produced is lower in *itchy* mice

Itchy mating pairs consistently had fewer pups than their wild type counterparts, and smaller litter sizes can sometimes be attributed to decreased sperm production in males. To test this, whole testes were collected and the total numbers of homogenization-resistant spermatid heads produced were counted from adult PND 56 C57BL/6J and *itchy* mice (Figure 3.3). Wild type C57BL/6J mice had an average of $1.2x10^7 \pm 3.2x10^5$ spermatid heads per testis counted, while the *itchy* mice averaged a significantly lower number of $0.9x10^7 \pm 4.7x10^5$ heads. Comparatively, this data indicate that *itchy* mice have a 27% reduction in total spermatid heads. Although this is statistically significant, this reduction does not appear to be severe enough to cause the marked observed decrease in litter sizes[53-57].

3.2.4 Similar Sox9 counts indicate that the observed decrease in spermatid heads in *itchy* mice does not result from decreased Sertoli numbers

Sertoli cells divide and terminally differentiate during early fetal life, with the finite number being established prior to the first wave of spermatogenesis[58]. In the

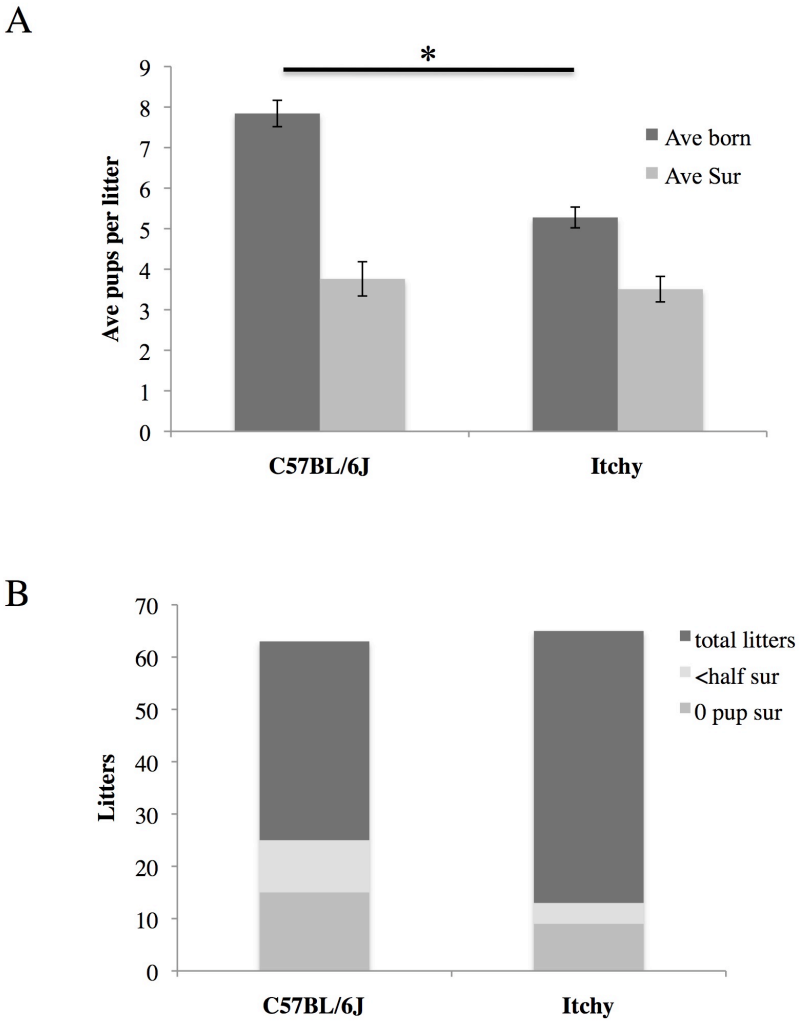


Figure 3.2 Pups per litter and survival rates of C57BL/6J and *itchy* breeding colonies.

A) The numbers of pups at day 0 and at day of wean/sacrifice were recorded. *Itchy* mice have smaller litter sizes that wild type, but their survival rates are similar. B) Analysis of the number of litters where less than half or none of the pups survived compared to the total number of litters. C57BL/6J mice had higher fatality rates. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

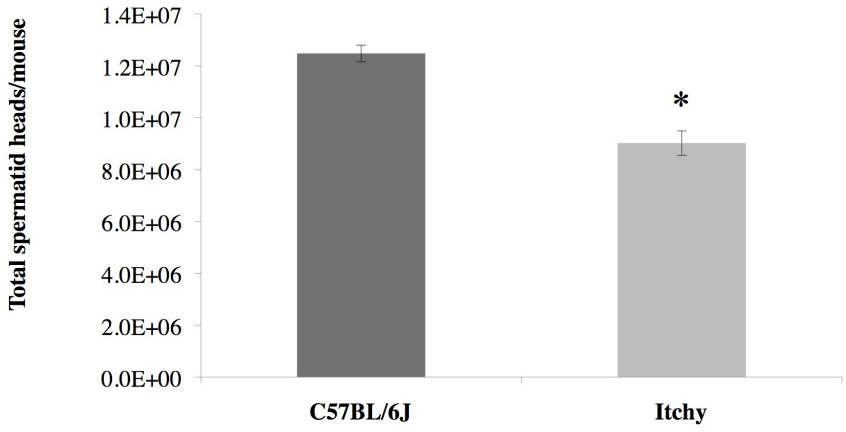


Figure 3.3 Spermatid head counts for C57BL/6J and *itchy* mice at PND 56.

Homogenization-resistant spermatid heads were counted from 8 mice from each genotype. *Itchy* mice produce significantly lower mature spermatid heads than their wild type counterparts. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

mouse, each Sertoli cell is able to support 30 to 50 developing germ cells[9, 10], and studies in which the numbers of Sertoli cells have been altered have shown that it is ultimately the number of Sertoli cells that defines the number of spermatids produced[12]. Therefore, the numbers of Sertoli cells per tubule were counted for the C57BL/6J and *itchy* mice at PND 28 and PND 56 (Figure 3.4). Although they show lower spermatid head counts, *itchy* mice have similar Sertoli cell numbers at both PND 28 (C57 24.2±0.6 and *itchy* 22.6±1.0) and PND 56 (C57 19.0±0.1 and *itchy* 17.8±0.0). This indicates that the decrease in spermatid head counts does not result from a decrease in the number of Sertoli cells. And although this suggests that Sertoli cell division and differentiation is most likely not altered by the loss of Itch, the support they provide the developing germ cells may still be affected.

3.2.5 Smaller litter sizes most likely result from a defect in embryo development rather than parental gamete formation

Cross-mating experiments were conducted to determine if the smaller litter sizes could be attributed to reproductive problems in the males or females. *Itchy* females were bred to C57BL/6J males and *itchy* males were bred to C57BL/6J females for 4 consecutive litters, and the total number of pups born was counted (Figure 3.5). Although the *itchy* females did produce smaller litters than the C57BL/6J females (*itchy* female 6.60±0.47 and C57 female 8.09±0.48), these numbers did not account for the significantly smaller litter sizes observed in the homozygous *itchy* colonies. This suggests a possible failure in $Itch^{-/-}$ pup development that is not observed in the $Itch^{+/-}$ offspring.

3.2.6 *Itchy* females have a greater number of embryo implantation sites than live pups born

Cross-mating experiments indicated that Itch may play an important role during embryonic development, and to examine this further, the number of embryo implantation

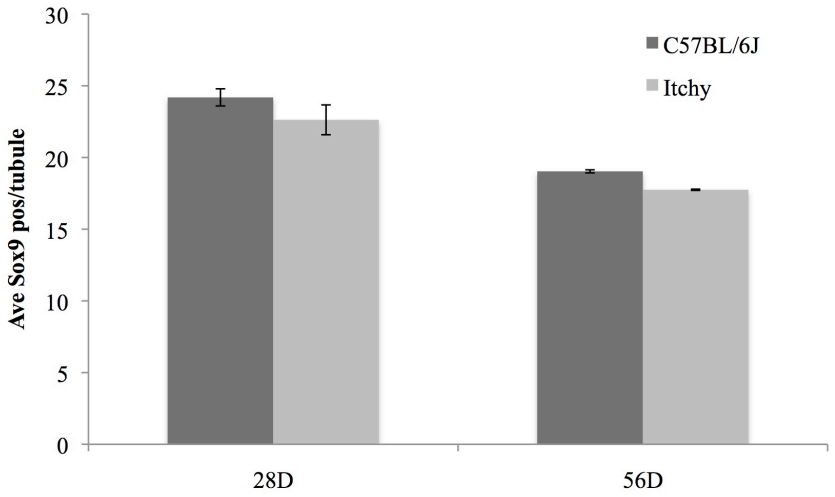


Figure 3.4 Quantification of Sox9-positive cells per tubule in C57BL/6J and *itchy* mice.

Immunohistochemistry was used to stain and quantify the number of Sox9-positive Sertoli cells per essentially round tubule per section. *Itchy* mice appear to have normal Sertoli cell development. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

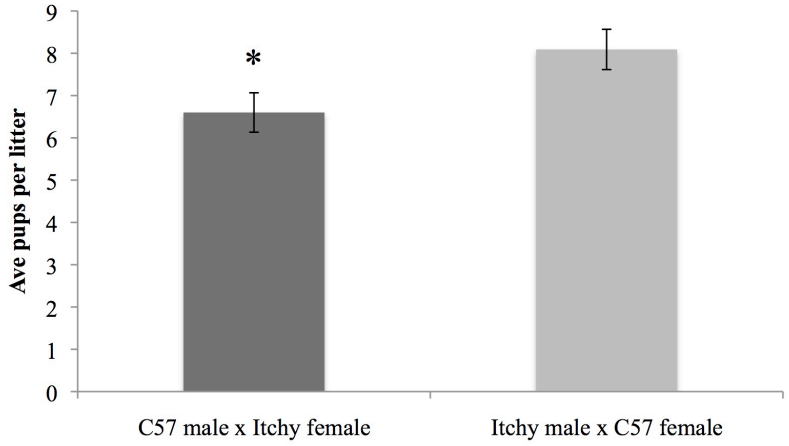
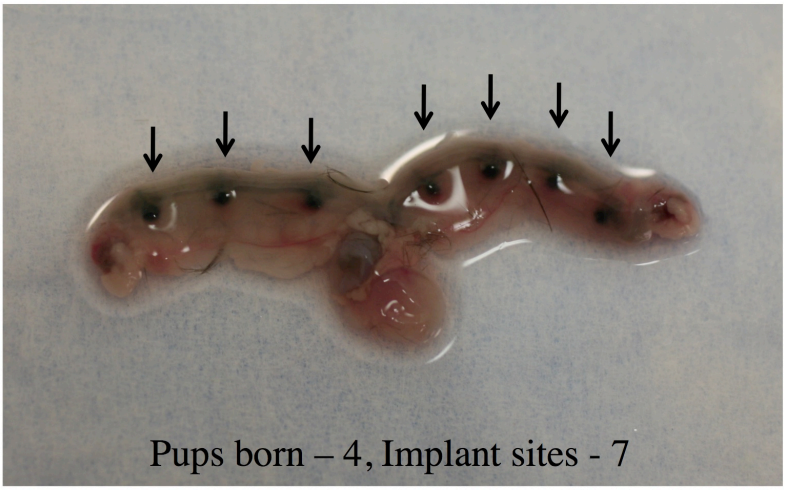


Figure 3.5 Cross-breeding experiments between C57BL/6J and *itchy* mice.

Itchy females were bred to C57 males and *itchy* males were bred to C57 females for four consecutive litters and the numbers of pups at day 0 were recorded. Although *itchy* females have slightly smaller litters than C57 females, they did not account for the significantly smaller sizes of the *itchy* colonies. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

sites in the uterine horns were quantified and compared to the number of pups born. This procedure takes advantage of the fact that the embryo sites turn black in a 10% ammonium sulfide solution, and therefore can be manually counted. Because embryo implant sites leave permanent scars, virgin female C57BL/6J and *itchy* mice were mated with males of the same genotypes and left undisturbed throughout pregnancy. Five days after birth, the females were sacrificed by CO_2 inhalation, and the uterine horns were dissected out and fully submerged in a 10% ammonium sulfide solution for 1 hour. After this time they were removed and placed in a petri dish, and the total number of black spots were quantified (Figure 3.6A). As described earlier, the *itchy* repeatedly have smaller litter sizes than their wild type counterparts (Figure 3.6B). Quantification of the embryo implantation sites revealed that they also have fewer fertilized eggs that attach to the uterine wall. Interestingly, when you compare the number of pups born to the number of implantation sites, the *itchy* mice show a significant loss in offspring following implantation, with only 67.5% of the embryos surviving until birth compared to 95% for the C57BL/6J. This suggests that Itch may play a minor role in female gamete production, but that it is necessary for proper fetal development.

A



B

		Pups Born	Implant sites	Pup/Site	Percent
Itchy+/+		9	9	1	100
		8	10	4/5	80
		9	9	1	100
		8	8	1	100
	Average	**8.5**	**9**	**19/20**	**95**
Itchy-/-		5	8	5/8	62.5
		6	7	6/7	85.7
		4	7	4/7	57.1
		4	7	4/7	57.1
		6	8	3/4	75.0
	Average	**5**	**7.4**	**2/3**	**67.5**

Figure 3.6 Quantification of C57BL/6J and *itchy* embryo/uterine implantation sites.

First time mothers were sacrificed five days after giving birth and their uterine horns were stained with 10% ammonium sulfide. A) Sites of embryo implantation appear as black spots. B) The number of pups born was compared to the number of implantation sites. *Itchy* mice have fewer implantation sites and loss a significant number of pups.

3.3 DISCUSSION

Much progress into deciphering the role of Itch has come from work in the immune system and the late onset autoimmune disease that occurs in the *itchy* mice, while its role in other tissues and systems remains less understood. As of now, this is the first and only study that not only examines these animals for their possible reproductive phenotype, but also analyzes them at a younger developmental age, before the onset of their classical disease. Even as early as PND 28, *itchy* mice have decreased body weights compared to wild type mice (Figure 3.1), and while at PND 56 their testis weights are lower, their testis to body weight ratios are consistently higher, indicating that these mice are overall smaller than C57BL/6J and that the body weight is more severely impacted by the loss of Itch than the testis weight.

The number of pups born per litter to the *itchy* mating pairs was almost half of that compared to the wild type mating pairs, although interestingly the average number of pups that survived until wean were nearly the same (Figure 3.2). A further look into the survival rate of the wild type mice revealed that the C57 litters were more likely to have less than half or none of the pups survive until the age of weaning. The cause of each case varied and was usually unknown, but this could possibly be attributed to the care given by the mothers. To analyze the source of the discrepancy in the number of pups born, various aspects of testicular function were examined and mating experiments were performed. Although male *itchy* mice display a lower numbers of mature spermatids (Figure 3.3), this decrease did not appear to be significant enough to result in the observed decrease in litter sizes. Male mice produce far more gametes than are required, so small declines in production are often unseen in fertility studies[53-57]. Sertoli cell counts, as measured by Sox9 staining (Figure 3.4), were similar in *itchy* mice as they

were in their wild type counterparts, revealing that this also did not account for the decrease in spermatid head counts.

Cross-mating experiments with wild type C57BL/6J females revealed no observable differences in their reproductive ability or number of viable offspring produced (Figure 3.5). As mentioned earlier, this is a very common finding in male reproductive disorders that cause lower sperm numbers, as many more sperm are produced than are actually required for fertilization[53-57]. Next we tested to see if there was a reproductive issue with the female *itchy* mice, but similar mating experiments to wild type C57BL/6J males did not replicate the significantly decreased litter sizes seen in the *itchy* colonies. It is quite possible that Itch is important in the female reproductive tract or during pregnancy, but it does not fully explain the observed phenotype. This led us to consider that there may be a problem during development of the *itchy* pups that does not occur in the *itchy* x C57BL/6J heterozygous offspring. To examine this further, we looked for possible fetal resorptions in the *itchy* mice by staining for implantation sites. Not only were there less implantation sites in the uterine horns of the *itchy* mice compared to wild type C57BL/6J mice, but they also showed a significant loss in pups (Figure 3.6). This suggests that some of the *itchy* fetuses are failing to develop properly or are being lost prior to birth.

Chapter 4: Histological Evaluation of the Testis of Pubertal and Adult *Itchy* Mice

4.1 Introduction and Rationale

Spermatogenesis is the process in which stem germ cells located at the basement membrane of the seminiferous epithelium move across the Sertoli cell blood-testis barrier towards the tubule lumen, dividing and differentiating to become mature sperm. The earliest of these stem germ cells, the spermatogonia, are unique in that they must go through two types of divisions: a self renewal division to replenish the population of stem cells and a differentiating process to ultimately form mature sperm[14, 15]. Differentiating preleptotene spermatocytes move across the blood-testis barrier, which is formed by tight junctions between neighboring Sertoli cells. These cells undergo two meiotic divisions to become haploid germ cells, called spermatids (reviewed in[16]). Spermatids then undergo significant morphological and cellular changes in order to modify their structure from the early round cell shape to the elongated and distinct form of a sperm cell. Round spermatids differentiate to produce elongating spermatids, and upon maturation are released into the lumen of the tubule. The release of sperm from the seminiferous epithelium marks the end of one cycle of spermatogenesis, which in a mouse takes about 35 days to complete[17]. The specific timing and synchronization of the different divisions allows for the tubules to be divided in to specific stages, 12 in the mouse, based on the germ cell subtypes that are present[19]. This can be a useful tool histologically, because alterations in spermatogenesis can lead to disruptions in tubule-stage dynamics, leading to visible and possibly even quantifiable differences.

Observable changes in morphology are an indicator of underlying testicular disease, and the cause of these changes can result in an outward testicular phenotype.

Loss of the integrity of the Sertoli cell-derived blood-testis barrier can cause premature or failure in release of the mature sperm, which could alter spermatid head counts[40]. Alterations in other junctional sites, such as between neighboring germ cells or between germ cells and Sertoli cells, can lead to clumping or disorganization of the cell layers. Altered germ cell division or disruptions in Sertoli cell phagocytosis can lead to multinucleated germ cells or apoptotic bodies that are left behind in the tubules[59, 60]. All of these changes might present as symptoms in a mouse model, and would be morphologically visible by examining the histology of testicular cross sections. Although the fertility issues that were examined in the previous chapter appeared to arise from a fetal and/or maternal issue, there were still observable and measureable alterations in the testes of the *itchy* mice at both PND 28 and PND 56 that remained unexplained.

The goal of the work presented in this chapter was to examine the gross histology of the testes of the *itchy* mice at PND 28 and PND 56 compared to that of their wild type C57BL/6J counterparts. The method used to evaluate the testis of these mice here was periodic acid-Schiff-hematoxylin (PAS-H) staining of 5μm paraffin-embedded testicular sections, a specific histology method that uses a combination of periodic acid and Schiff's reagent to label the glycogen on spermatid acrosomes pink and hematoxylin to label the nuclei purple. Interestingly, *itchy* mice at PND 28 show an increase in the number of seminiferous tubules with meiotic figures and a delay in spermatogenesis. Also noticeable at this age is the appearance of irregular dividing/arrested cells at the basement membrane of several of the tubules. At PND 56, *itchy* mice continue to have the meiotic phenotype observed at PND 28, but they also show a series of distinct alterations, suggesting an age-dependent role for Itch in the testis. Alterations in spermatid head formation and organization in the *itchy* mice may be the underlying cause of the decreased head counts previously observed.

4.2 RESULTS

4.2.1 Testicular histology of peri-pubertal (PND 28) *itchy* mice reveals alterations in meiosis and a delay in spermatogenesis

In order to further elucidate the role of the E3 ligase Itch during normal testis development, testicular cross sections were stained and histologically evaluated. At PND 28, *itchy* mice have more tubules with meiotic figures than wild type C57BL/6J mice. (Figures 4.1A-D). Quantification of essentially round tubules revealed that wild type mice have an average of 7.4 ± 0.4% tubules containing meiotic germ cells, while *itchy* mice have a significantly higher average of 10.7 ± 0.3% (Figure 4.2A). Also notable in wild type mice at this age is the formation of mature spermatid heads, which can act as an indicator of normal physiology. Although C57BL/6J mice at PND 28 have completed the first cycle of spermatogenesis and contain elongated spermatids (Figure 4.1E), the *itchy* mice appear to be developmentally delayed. Comparing similar tubule stages, mature spermatid subtypes that are present in wild type mice are lacking in the *itchy* mice (Figure 4.1G). This could result from the altered meiosis that is also observed, where a change in cell cycle dynamics may alter the normally identifiable stages. Also possible is a delay in the initiation of the cell cycle, and therefore spermatogenesis, possibly by a late onset organization of the blood testis barrier.

4.2.2 Peri-pubertal *itchy* mice present with irregular dividing/arrested cells along the basement membrane of some tubules

Histological evaluation of the increase in stage 12 meiotic figures in the *itchy* mice also led to the discovery of another subset of irregularly dividing cells, this time at the basement membrane of the seminiferous epithelium. A closer examination of PAS-H-stained testicular cross-sections from PND 28 *itchy* mice revealed a series of tubules that contained what appeared to be cells either actively dividing or arrested near the basal

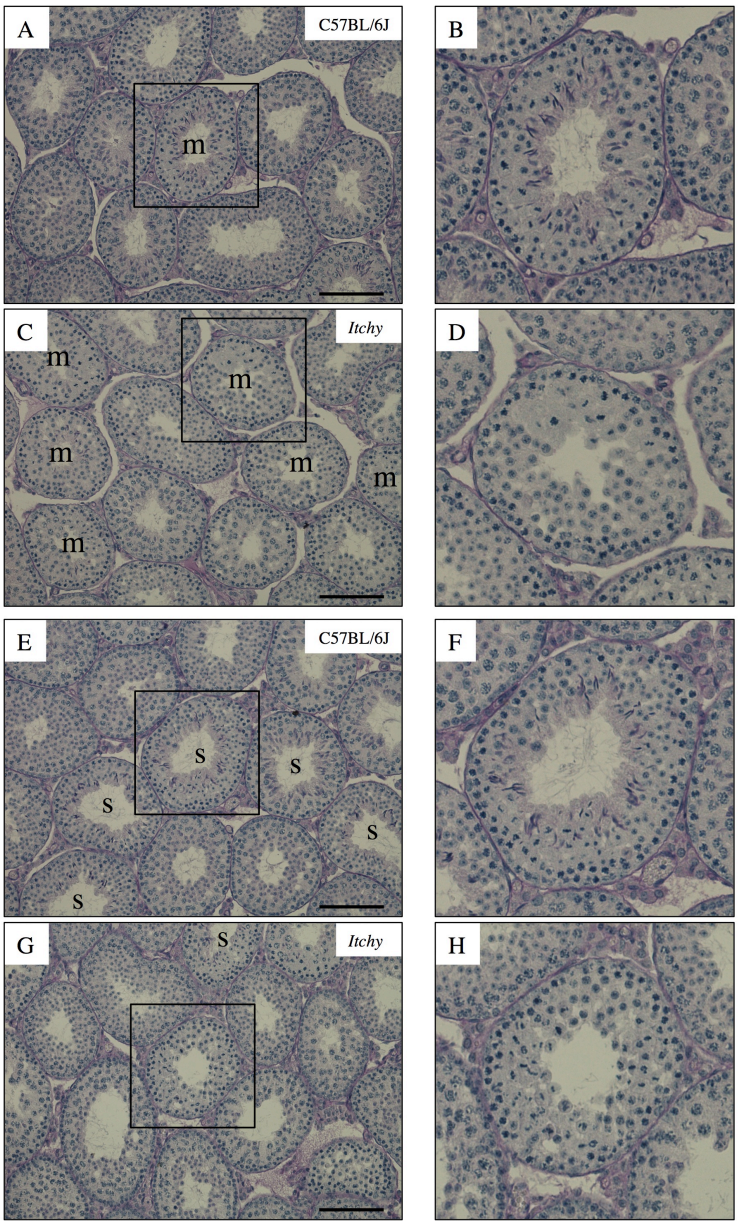


Figure 4.1 Histological evaluation of the testis of C57BL/6J and *itchy* mice at PND 28.

Testicular cross sections from (A,E) C57BL/6J and (C,G) *itchy* mice were examined using PAS-H staining. The box indicates the area magnified to the right (B, D, F and H). *Itchy* mice have more tubules with meiotic figures (C, denoted "m") and fewer tubules with mature spermatids (G, denoted "s"). The bar represents 100μm.

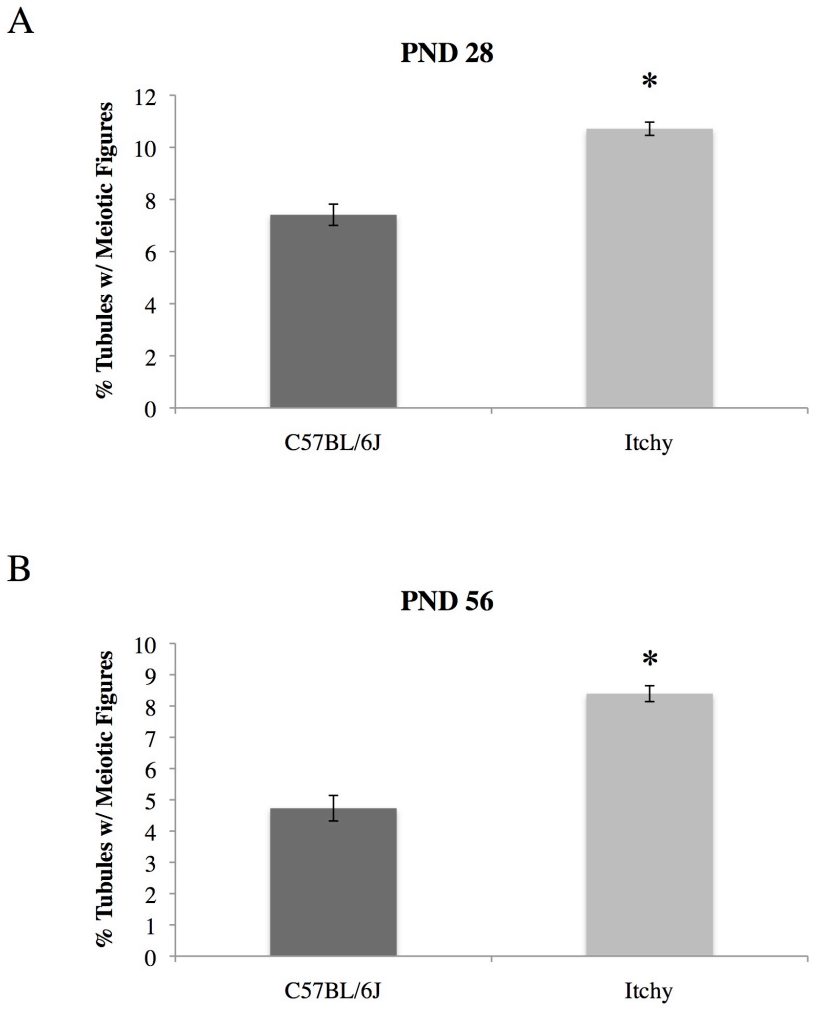


Figure 4.2 Quantification of tubules with meiotic figures in C57BL/6J and *itchy* mice.

The numbers of essentially round tubules containing meiotic figures at A) PND 28 and B) PND 56 were quantified as a percentage of the total number of tubules. *Itchy* mice have a higher percentage of tubules containing meiotic figures at both ages compared to wild type C57BL/6J mice. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

lamina (Figures 4.3A-F). These cells showed negative staining on both TUNEL and cleaved caspase 9 immunohistochemistry analysis, indicating that they are not apoptotic cells that have been phagocytosed by the supportive Sertoli cells (data not shown). Several interesting questions initially arise about these cells, including whether they are mitotically or meiotically active, what the germ cell subtype is, and whether they are arrested or just actively dividing. There does not appear to be a stage-specificity to their presence (Figure 4.3), which may be more likely with meiotically arrested spermatocytes. As for the specific subtype, immunohistochemical analysis using the early spermatogonial marker FoxO1 was negative, which rules out the early stage spermatogonia (Figure 4.4), but still leaves the possibility of later stage spermatogonia and early spermatocytes.

4.2.3 Testicular histology of adult (PND 56) *itchy* mice shows an increase in residual cellular material and disruptions in the germ cell layers

To determine if the alterations observed at PND 28 were age specific effects due to the loss of Itch, testicular cross sections from mice at PND 56 were also examined (Figures 4.5-4.7). The presence of mature spermatids in the *itchy* knockout mice suggests that the developmental delay observed at PND 28 did not have a long lasting effect on spermatogenesis, but the meiotic phenotype remained (Figure 4.2B), possibly pointing to an age-independent target of Itch. Cross sections from the *itchy* mice at PND 56 still contained a number of abnormalities, albeit different from those seen at PND 28, mainly because they were focused around development of the elongating spermatids. There appears to be an increase in the number of abnormal clusters of cells present in the testis (Figure 4.5B), although the cause remains unclear. These figures do not appear to be multinucleated germ cells, but rather clumps or large masses of cellular material, possibly leftover residual cytoplasm from released spermatids. They lack stage specificity (Figures

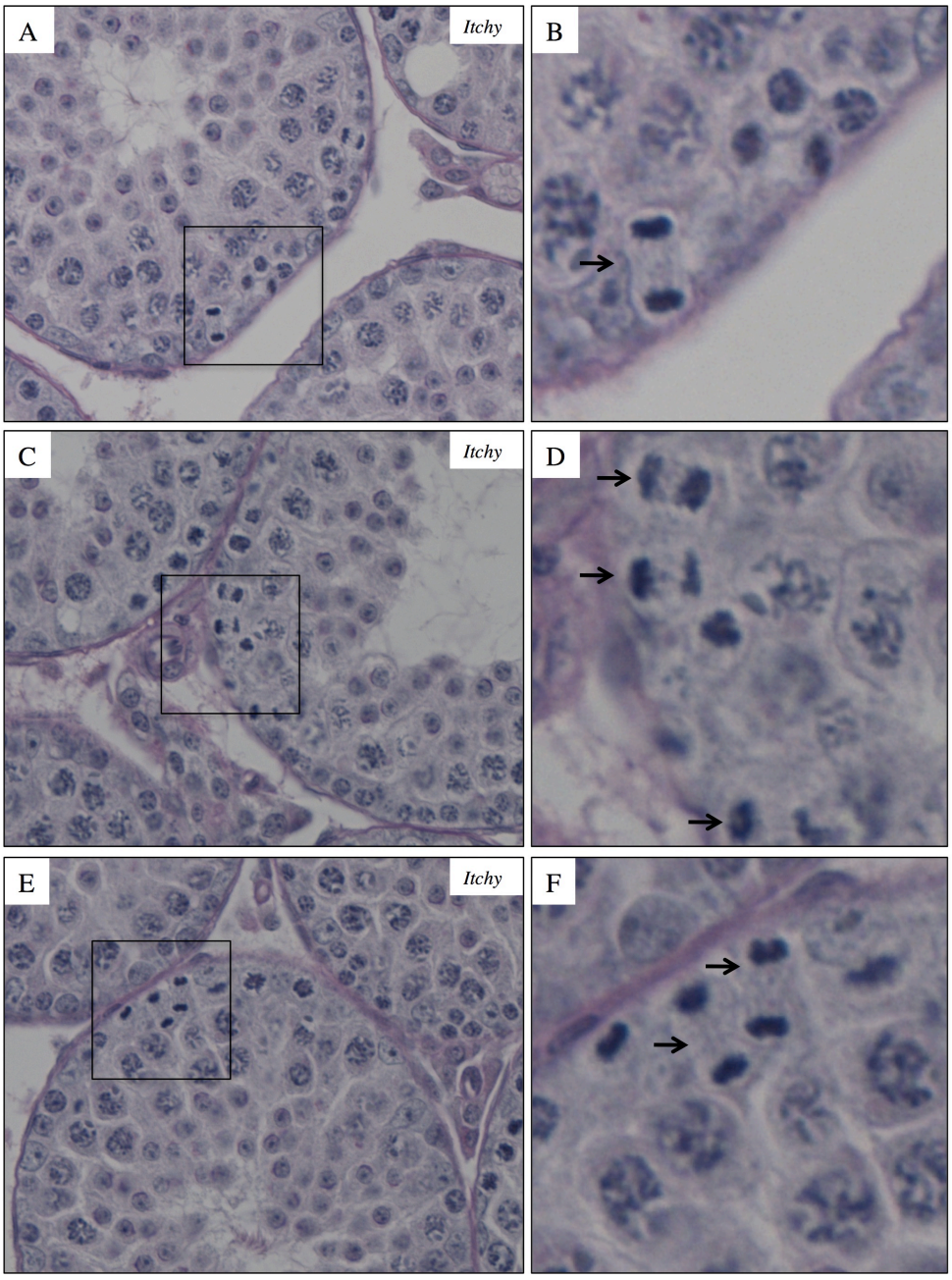


Figure 4.3 Some *itchy* tubules contain unusual dividing cells at the basement membranes.

Testicular cross sections (5μm) stained with PAS-H from PND 28 *itchy* mice revealed a subset of irregularly dividing mitotic/meiotic cells (arrows) near the basement membranes of some tubules. The box indicates the area magnified to the right.

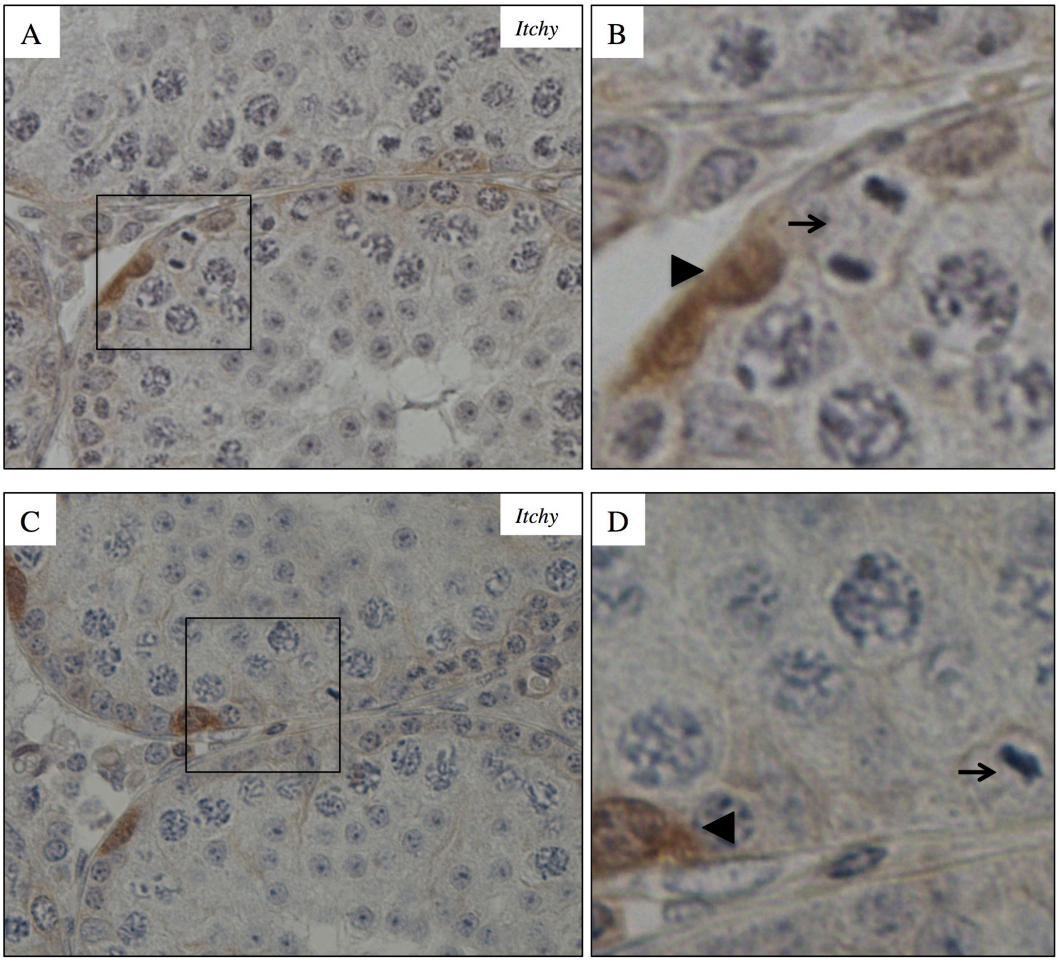


Figure 4.4 Immunohistochemical analyses of spermatogonia in PND 28 *itchy* mice.

Immunohistochemistry was used to specifically label early spermatogonia using a FoxO1 antibody. The box indicates the area magnified to the right, arrows point to cells of interest, and arrowheads point to FoxO1 positive cells. Dividing cells are negative for FoxO1.

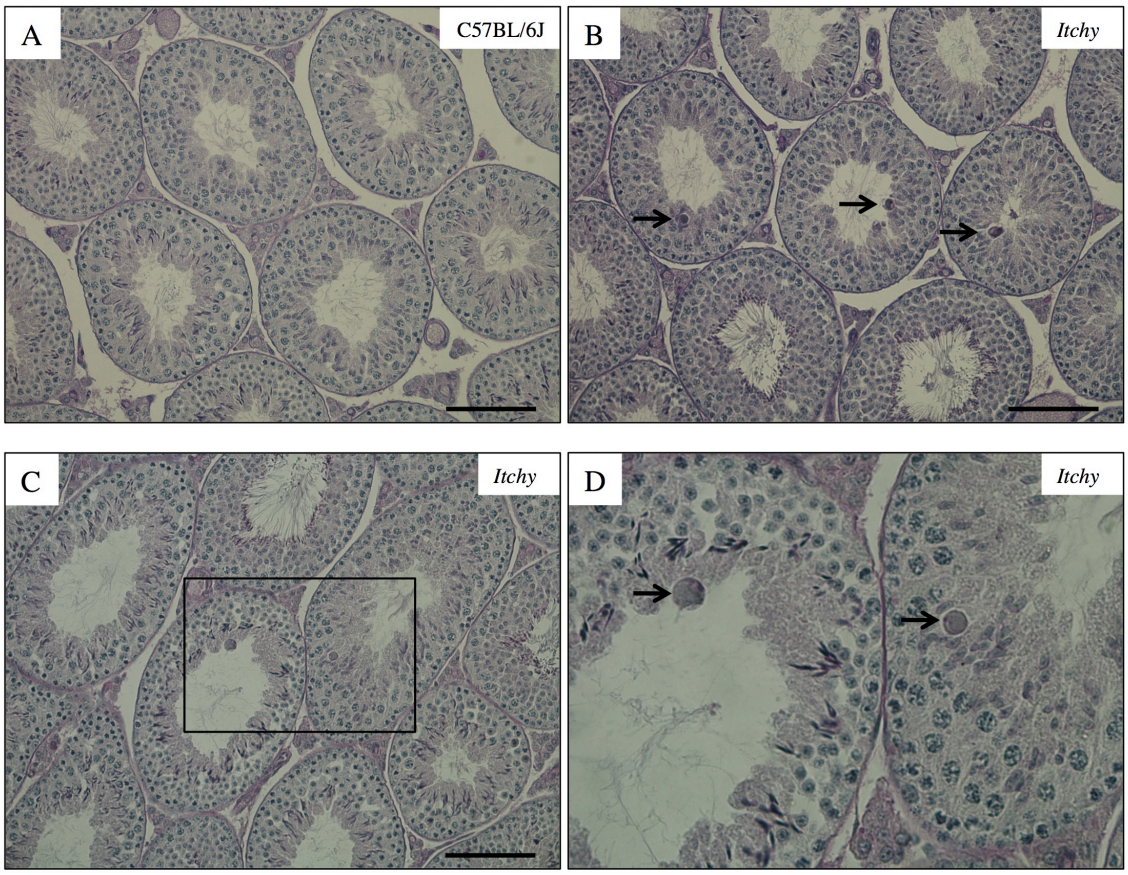


Figure 4.5 Histological evaluation of the testis of C57BL/6J and *itchy* mice at PND 56.

Testicular cross sections from (A) C57BL/6J and (B,C,D) *itchy* mice were examined using PAS-H staining. At PND 56, *itchy* mice show an increase in abnormal cells (A) compared to wild type (B), and they do not appear to be stage specific (C). The box indicates the area magnified to the right, and arrows point to sites of described abnormal histology. The bar represents 100μm.

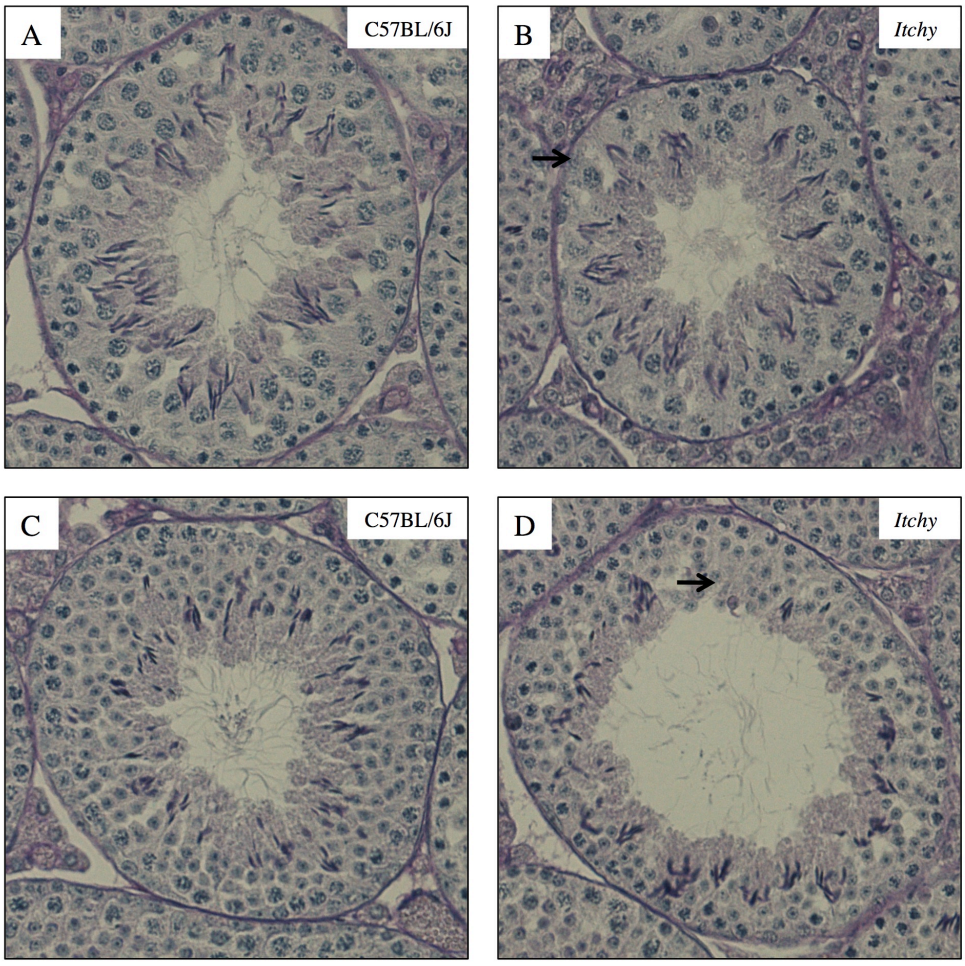


Figure 4.6 Histological evaluation of the testis of C57BL/6J and *itchy* mice at PND 56.

Testicular cross sections from (A,C) C57BL/6J and (B,D) *itchy* mice were examined using PAS-H staining. At PND 56, *itchy* mice have an increased number of tubules that lack distinct germ cell subtypes in both early (D) and late (F) stages, characterized by gaps in the normally continuous "ring" of germ cell stages within the tubule. Arrows point to sites of described abnormal histology.

4.5C-D), but may indicate a disruption in Sertoli cell phagocytosis that leads to their long-term retention. Observations of these clumps in TUNEL-stained and cleaved caspase 9 IHC slides show negative results, further supporting this hypothesis (data not shown). Along with this increase in leftover cellular material, some tubules show disrupted germ cell layers, where gaps in the germ cell layers appear in both early (Figure 4.6B) and late (Figure 4.6D) seminiferous tubule stages. This phenotype is indicated by a discontinuity in the observed "ring" of a distinct subset of germ cells in essentially round cross sections of seminiferous tubules, and gaps in early cell types my lead to missing late stage spermatids in the same regions. These irregularities were not as frequently observed in the wild type C57BL/6J mice (Figures 4.5A, 4.6A,C).

4.2.4 Adult *itchy* mice have alterations in elongate spermatid head formation and organization

Further histological examination of the PND 56 *itchy* mice revealed a disruption of the later stage mature elongate spermatids (Figure 4.7). In wild type C57BL/6J mice, elongate spermatids are positioned perpendicular to and uniformly around the lumen of each tubule (Figures 4.7A,C). The *itchy* mice, however, show a variety of subtle alterations in this typical pattern, including elongate spermatids that are orientated parallel to the lumen rather than perpendicular (Figure 4.7B) and appear to be clustered together and not uniformly arranged in the tubule (Figure 4.7D). Although the role of Itch during testis development has not yet been identified, these observations suggest that Itch is important in normal spermatogenesis, possibly during spermatid organization and maturation, and that its role may be age dependent.

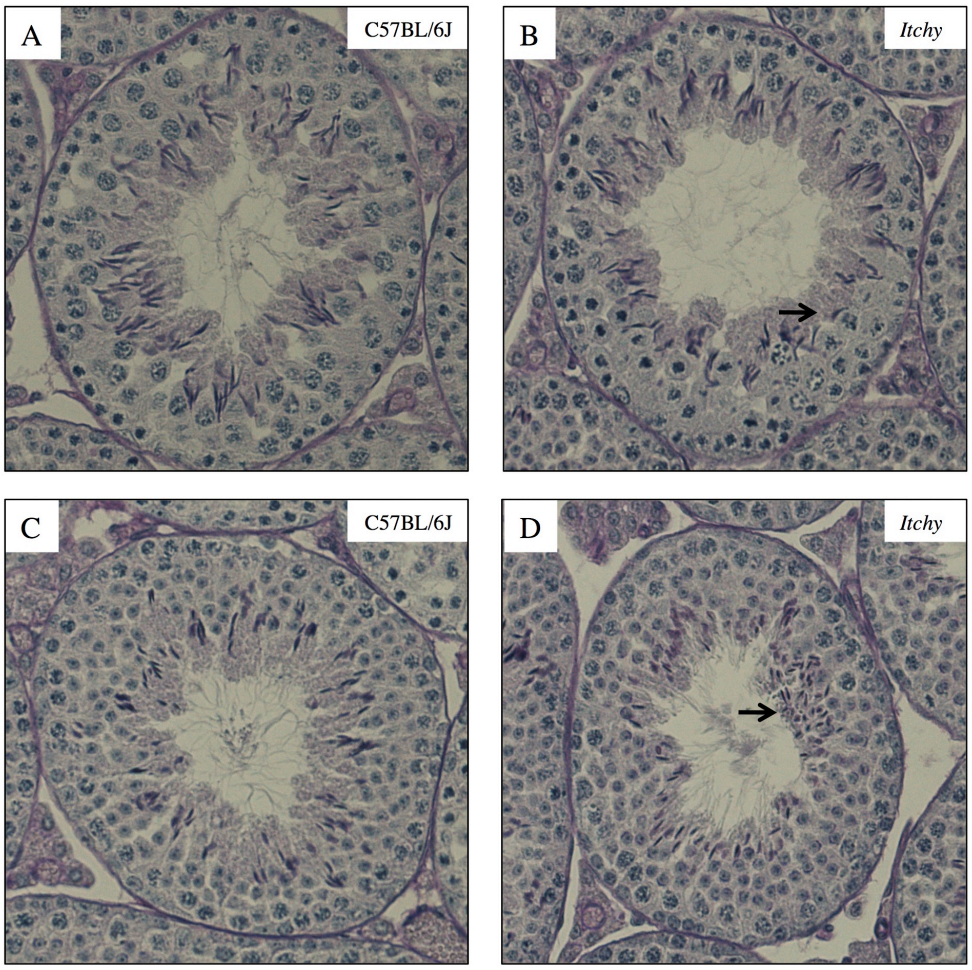


Figure 4.7 Spermatid head organization in C57BL/6J and *itchy* mice at PND 56.

Testicular cross sections from (A,C) C57BL/6J and (B,D) *itchy* mice were examined using PAS-H staining. *Itchy* mice show alterations in spermatid head formation, including disorientated and disorganized spermatids. Arrows point to sites of described abnormal histology.

4.3 DISCUSSION

Histological analysis of the *itchy* mice revealed several subtle although consistent changes throughout the seminiferous epithelium. At PND 28, the loss of Itch seems to influence germ cell division, with *itchy* mice having more tubules with meiotic figures and less tubules with late stage spermatids (Figure 4.1). It is unknown whether meiosis is permanently arrested or whether the length of the cycle is extended, but the lack of spermatids points to a clear delay in normal spermatogenesis. It also remains unclear whether these two phenotypes are linked, or if they are independent responses to the loss of Itch. For example, Itch may play dual roles in both the cell cycle and the blood-testis barrier (BTB), and therefore its loss may result in a delayed organization of the BTB, which would delay the initiation of the spermatogenesis and therefore spermatid formation, and possibly extend the length of the cell cycle.

Interestingly, along with the observed increase in meiotic events, PND 28 *itchy* mice also appear to have a series of early stage germ cells actively dividing near the basement membrane of the seminiferous epithelium (Figure 4.3). Histological examination of these cells suggests that they are mitotically active, and possibly a late stage spermatogonial subtype, based on their lack of FoxO1 staining (Figure 4.4), although it remains unclear whether they are permanently arrested or perhaps delayed within the cycle. This may point to a role for Itch in germ cell mitosis as well as meiosis, and in fact some of the previously identified targets of Itch have been generally linked to the cell cycle.

At PND 56, the *itchy* mice are able to produce mature and functional sperm, suggesting that they can at least recover from the delay, although they still show alterations in meiosis. Itch appears to take on a different role during adulthood; with PND

56 *itchy* mice showing a series of spermatid head defects and a disorganization of the cell layers (Figure 4.6-4.7). The large masses of cellular material left over in various stages of the seminiferous epithelium possibly indicate a failure of Sertoli cells to properly dispose of spermatid cytoplasm (Figure 4.5). The observed alterations of the late stage elongate spermatids may explain the decreased spermatid head counts as clumped material would less likely be counted. Although these phenotypes appear subtle, the combination of several smaller issues may lead to the more significant issues such as increased apoptosis, decreased testis weights, and decreased spermatid head counts.

Chapter 5: Altered Germ Cell Apoptosis in *Itchy* Mice at PND 28 and PND 56

5.1 Introduction and Rationale

Apoptosis, or programmed cell death, is an important mediator of spermatogenesis, and is used to both eliminate damaged germ cells and to limit the germ cell population to the supportive capacity of the Sertoli cells. There are two major apoptotic pathways: extrinsic, or death receptor-mediated apoptosis and intrinsic, or mitochondrial-mediated apoptosis. In the testis, intrinsic apoptosis occurs when germ cells undergo cellular stress, such as DNA damage, and mitochondrial signaling leads to the release of cytochrome c, which binds to Apaf1 and caspase 9 to form the apoptosome (intrinsic apoptosis reviewed in[21]). This results in the cleavage of caspase 9, initiating a cascade of caspase cleavage events that ultimately results in apoptosis[22]. Extrinsic apoptosis, however, is typically thought of as being initiated by Sertoli cells, which must maintain and limit the number of germ cells to match their own supportive capacity. Death ligands are expressed on the surface of Sertoli cells, and binding to their cognate receptor on the surface of germ cells, causing receptor clustering[23-25]. This recruits adaptor proteins within the germ cells, and the initiator caspase 8, forming the death inducing signaling complex, or DISC, which results in the autocatalytic cleavage of caspase 8[26]. The cleaved form of caspase 8 then goes on to cleave and activate the downstream effector caspases, resulting in a similar apoptotic pathway as observed in intrinsic signaling[27].

Although Itch itself is not directly involved in either of the apoptotic pathways, several common proteins both upstream and downstream of the E3 ligase Itch are associated with apoptosis. For example, cellular FLICE-like inhibitory protein, or cFLIP,

is an inhibitor of caspase 8, and can prevent cleavage by binding the DISC and preventing caspase 8 binding. In recent studies, Itch has been shown to target and ubiquitinate cFLIP, labeling it for degradation by the 26S proteasome[45]. This prevents cFLIP from blocking caspase 8 cleavage and activation, therefore promoting apoptosis. The p53 transcription factor family member p73, which responds to DNA damage, is another protein that has been shown to be a target of Itch[61]. The stress kinase c-Jun N-terminal Kinase, also known as JNK, has been linked to both intrinsic and extrinsic apoptotic signaling, and several groups have shown that Itch activation requires JNK-dependent phosphorylation[45]. These several connections point to the idea that Itch may play a role in normal apoptosis, and suggest, therefore, that the loss of this protein in the *itchy* mice would result in a possible decrease in apoptosis.

The aim of this chapter was to examine the involvement of Itch during physiological germ cell apoptosis. To examine this, total apoptosis was evaluated through the use of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of paraffin-embedded testis sections, and western blot analysis and immunohistochemistry of factors involved in both intrinsic and extrinsic apoptotic signaling. Unexpectedly, *itchy* mice were found to have higher rates of basal apoptosis, both at a peripubertal PND 28 age and at an adult PND 56 age. Examination of extrinsic markers revealed no significant differences in the germ cells, while caspase 9 cleavage appeared to have an age-dependent relationship with Itch. These results, along with the meiotic phenotype described previously, point to a role for Itch in germ cell division, and therefore the disruption that occurs in the *itchy* mice results in an alteration in the intrinsic apoptosis pathway.

5.2 RESULTS

5.2.1 *Itchy* mice have a higher apoptotic index than wild type C57BL/6J mice at both peri-pubertal and adult ages

Itch has been linked to death receptor-mediated apoptosis through its targeted ubiquitination and subsequent proteasomal degradation of the anti-apoptotic protein cFLIP[45]. In order to determine if Itch plays a role in the normal physiological apoptosis of germ cells, testicular cross sections from *itchy* mice were examined at PND 28 and PND 56 (Figure 5.1). TUNEL analysis of wild type C57BL/6J mice reveals an apoptotic index typical of normal, background apoptosis (6.91±0.58% at PND 28 and 4.12±0.34% at PND 56). Although it was predicted that a decrease in apoptosis would be observed with the loss of this pro-apoptotic protein, *itchy* mice actually show an increase in apoptosis in both the peri-pubertal PND 28 (8.85±0.43%) and at the adult PND 56 (7.20±0.50%) mouse testis. This represents an increase if approximately 28% at PND 28 and 75% at PND 56 compared to wild type C57BL/6J mice.

5.2.2 Western blot analysis revealed no significant changes in extrinsic apoptosis markers within the germ cells

Histological analysis of the incidence of germ cell apoptosis revealed that the *itchy* mice have higher basal levels of apoptotic germ cells than their wild type counterparts, but the mechanism behind this observed increase is not apparent. Therefore, the two major pathways of apoptosis were examined using western blot analysis and immunohistochemistry. Extrinsic apoptosis, or death receptor-mediated apoptosis was evaluated by assessing the levels of two death ligands, FasL and TRAIL, and their cognate receptors, Fas and DR5, previously identified in our lab as contributing to the regulation of germ cell apoptosis (Figure 5.2A). The two ligands were expressed at

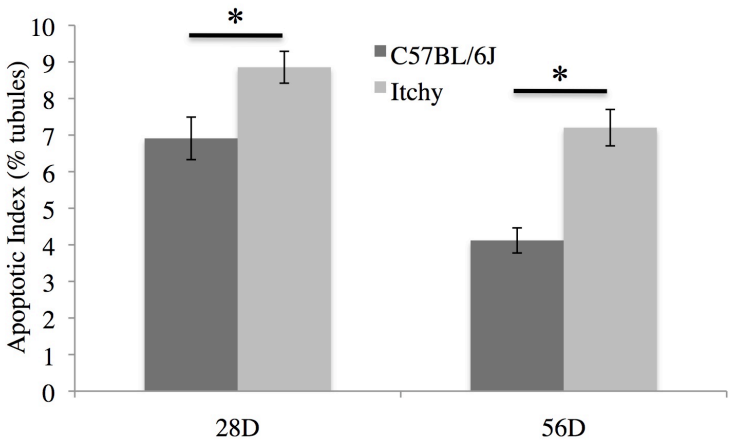


Figure 5.1 Testicular apoptotic indexes of C57BL/6J and *itchy* mice at PND 28 and PND 56.

Apoptosis was quantified using testicular cross sections and TUNEL assay. The apoptotic index was calculated as the percentage of essentially round tubules containing more than 3 TUNEL-positive germ cells. *Itchy* mice have a higher apoptotic index than wild type mice at both of the ages examined. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

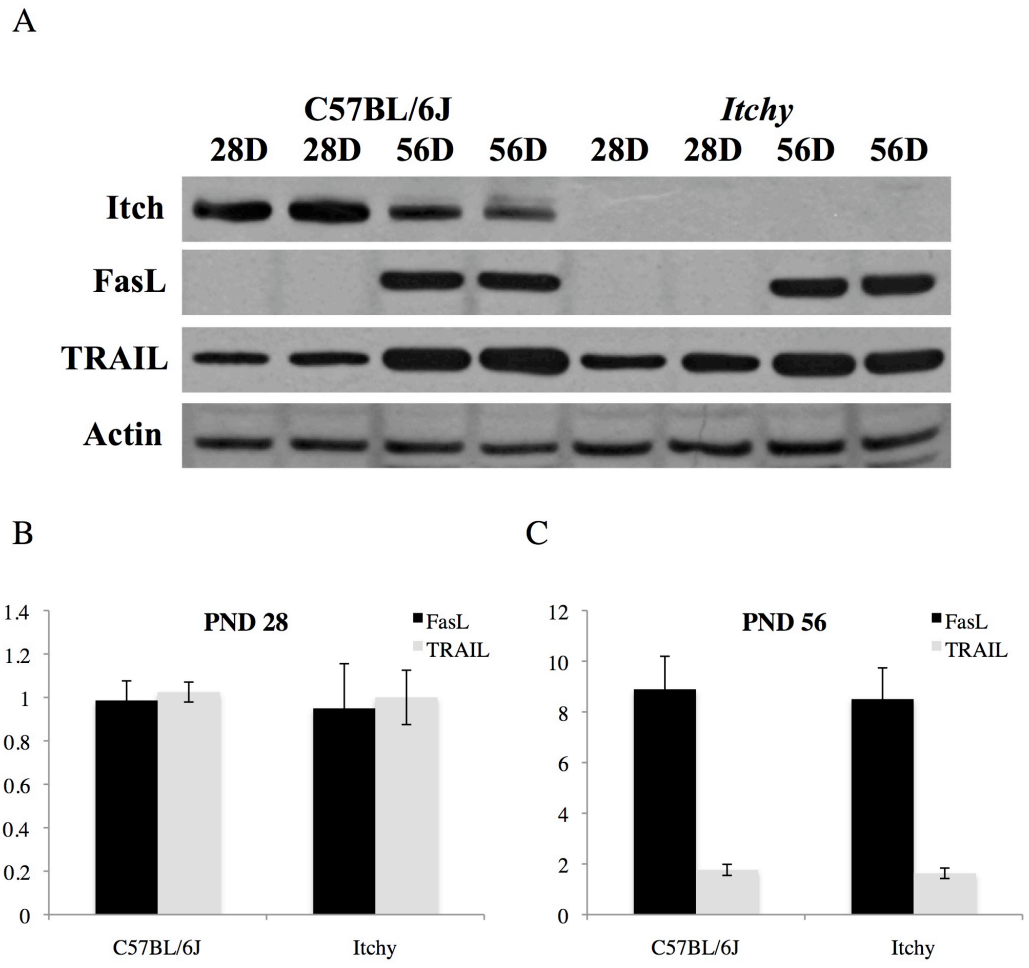


Figure 5.2 Western blot analysis of the death ligands in C57BL/6J and *itchy* mice at PND 28 and PND 56.

A) Total protein from whole testis tissue was analyzed using western blot analysis and primary antibodies against Itch, FasL, and TRAIL. Actin was used as a loading control. Quantification at B) PND 28 and C) PND 56 was performed using ImageJ (NIH). As expected, *itchy* mice are deficient in Itch protein, but no significant differences are seen in the targets between the genotypes. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

higher levels at PND 56 in both the wild type and *itchy* mice, although they were expressed at similar levels between the two genotypes (Figure 5.2B-C). As for the receptors, no obvious changes were seen in DR5, however there was a significant increase in Fas in the *itchy* mice at PND 28 that disappeared at PND 56 (Figure 5.3A). To narrow down the cellular source of this difference, seminiferous tubules were isolated from PND 28 C57BL/6J and *itchy* testes to remove the interstitial cells, and western blots were performed in comparison to whole testis preparations (Figure 5.3B). Removal of the interstitial material successfully removed the increase in Fas observed in the *itchy* mice, indicating that a source other than the germ cells, possibly the Leydig cells or infiltrating immune cells, are responsible for this discrepancy.

5.2.3 Immunohistochemical analysis of the intrinsic signaling pathway

Intrinsic apoptosis, or mitochondrial-mediated apoptosis, was analyzed by the immunohistochemical detection of the cleaved form of caspase 9 (Figures 5.4A-E). Interestingly, the loss of Itch appears to have an age-dependent effect on the cleavage of caspase 9 in germ cells, a linkage that has not been previously described (Figure 5.4E). At PND 28 (Figures 5.4A-B), wild type C57BL/6J mice have a basal level of 8.12±0.64% of tubules with more than three cleaved caspase 9 positive germ cells, while the age-matched *itchy* mice have a reduced level of 4.95±1.09%. This indicates that not only may Itch act to promote intrinsic apoptosis signaling, but that the increased apoptosis observed in the *itchy* mice most likely results from an extrinsic signaling mechanism. On the contrary, at PND 56 (Figures 5.4C-D), where the number of cleaved caspase 9 positive cells is reduced in the wild type C57BL/6J mice (3.81±0.76%) they are significantly increased in the *itchy* mice (8.53±1.23%), which corresponds to the overall increased apoptosis seen in these animals. Therefore, the loss of Itch leads to age-dependent

alterations in germ cell apoptosis, although the mechanisms that account for these differential changes remain unclear.

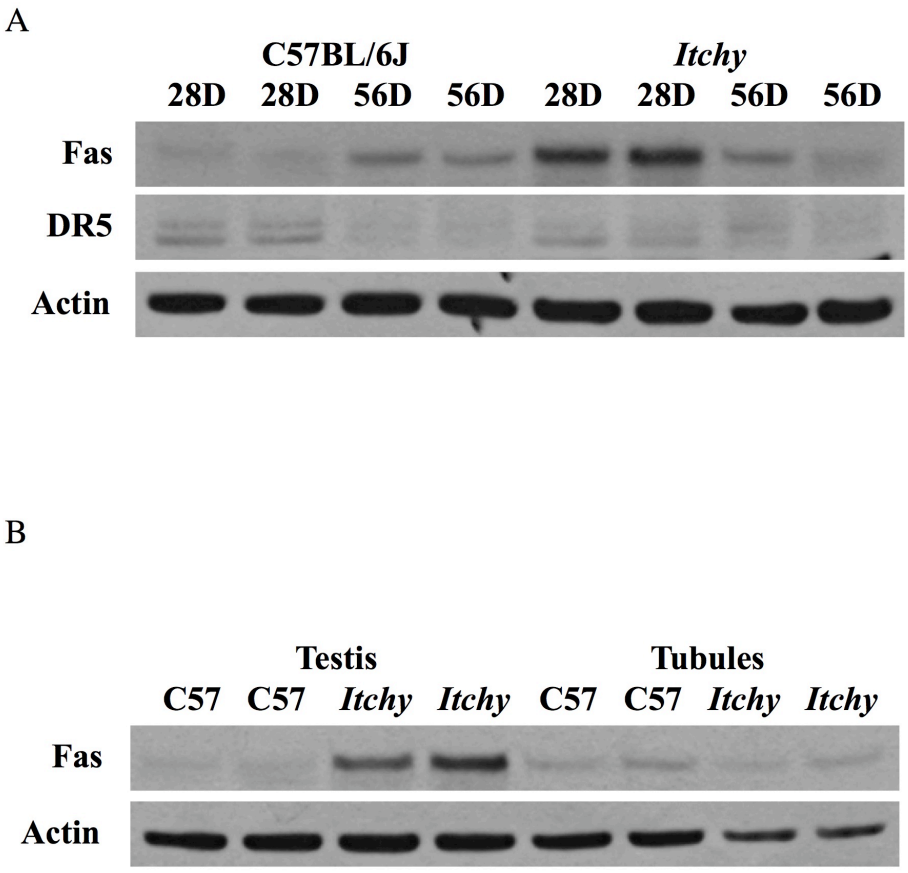


Figure 5.3 Western blot analysis of the death receptors in C57BL/6J and *itchy* mice at PND 28 and PND 56.

A) Total protein from PND 28 and PND 56 whole testis tissue was analyzed using western blot analysis and primary antibodies against Fas and DR5. B) Total protein from PND 28 whole testis tissue and tubule isolates were analyzed using western blot analysis and primary antibodies against Fas. Actin was used as a loading control. Fas is increased in the PND 28 *itchy* mice, but it is localized within the interstitial space.

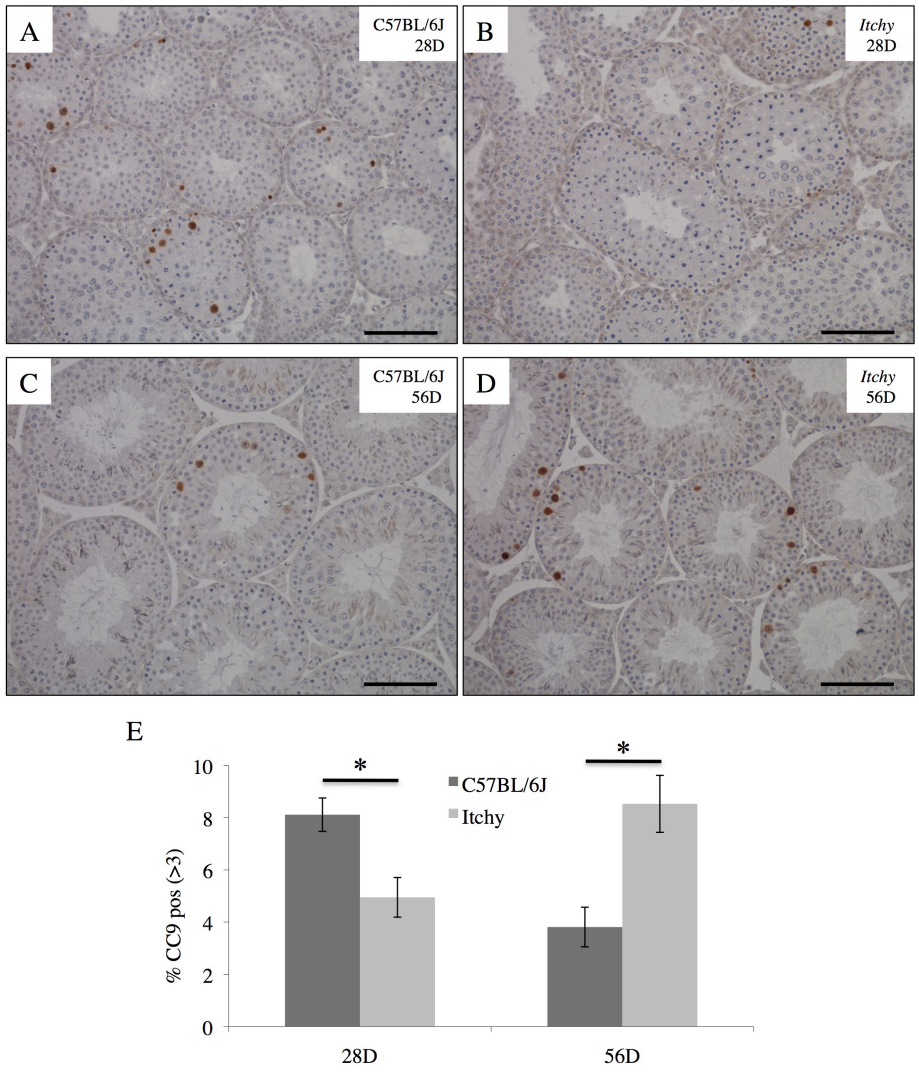


Figure 5.4 Immunohistochemical analysis of intrinsic apoptosis in C57BL/6J and *itchy* mice at PND 28 and PND 56

Immunohistochemical analysis was performed using an antibody specific for cleaved caspase 9 and testicular cross sections from (A,C) C57BL/6J and (B,D) *itchy* mice. The bar represents 100μm. E) The index was calculated as the percent of essentially round tubules that contained more than 3 positive cells. *Itchy* mice have lower intrinsic apoptosis at PND 28 and higher at PND 56. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

5.3 DISCUSSION

Although it was originally hypothesized that the *itchy* mice would have lower basal levels of apoptosis due to the predicted stabilization of the cFLIP protein, the exact opposite was in fact observed, with the *itchy* mice having significantly higher levels at both the of the ages examined (Figure 5.1). Interestingly, a similar increase in the incidence of apoptosis was seen in a mouse model that lacks the proapoptotic death ligand FasL[62]. The FasL gene deficient mice, which also display an autoimmune phenotype, have significantly higher levels of germ cell apoptosis than the *itchy* mice, but they show a similar pattern of delayed spermatogenesis and decreased spermatid head counts compared to wild type C57BL/6J mice. Unpublished observations from our lab of TRAIL gene deficient mice, another death ligand family member, show similar results, which may point to a general phenotype for disrupted germ cell apoptosis.

Death receptor signaling has not only been linked to Itch-mediated ubiquitination, but it also plays an important role in normal testis homeostasis, and since total apoptosis is disturbed in the *itchy* mice, the extrinsic signaling players were examined. Increases in death ligands can result in increased apoptosis, but protein expression analysis failed to reveal significant changes in either FasL or TRAIL in the *itchy* mice at either PND 28 or PND 56 (Figure 5.2). Increased receptor expression can also lead to alterations in apoptosis, and western blot analysis of Fas revealed significantly higher levels at PND 28 in the *itchy* mice (Figure 5.3). Although this initially appeared to explain our observed increase in apoptosis, tubule isolation and removal of the interstitial cells was able to abolish this difference, indicating that the increased Fas was not from a germ cell source. While this may not help to reveal the mechanism behind the altered apoptosis levels, this does pose an interesting question about the source of increased Fas. Within the

interstitium there are several resident cell types, including Leydig cells that normally express Fas, and there is the possibility of invasion by nonresident cell types, more significantly in the model of autoimmune disease. Animals that have over active immune systems can over produce increased amounts of immune cells, which can target and invade many tissues, including the testis, and these cells often express Fas. The specific cell type, along with the reason behind the age-dependent specificity of the increase, may play a role in the larger overall health of the animal.

Although we did not measure an increase in death receptor signaling within the seminiferous epithelium, we did observe a difference in the cleavage of caspase 9 and the interpreted activation of the intrinsic signaling pathway (Figure 5.4). C57BL/6J mice show a similar pattern of cleaved caspase 9 positive staining as they do TUNEL, with levels around 8% at PND 28 and around 4% at PND 56. This, however, is reversed in the *itchy* mice, which have lower levels of caspase 9 cleavage at PND 28 (~5%) and more than double at PND 56 (~8.5%) compared to wild type. Several inferences can be drawn from this apparent difference in apoptotic signaling. First, Itch appears to effect caspase 9 cleavage in an age dependent manner. At PND 28 in wild type mice, Itch may promote caspase 9 activation, and therefore in the absence of Itch there is less cleavage. On the other hand, at PND 56, Itch may switch roles, where its presence now inhibits caspase 9, causing significantly higher levels of cleavage in the *itchy* knockout mice. This falters, though, when these data are compared to the overall apoptotic indexes determined by TUNEL. At PND 56, the high levels of caspase 9 cleavage are able to account for the corresponding high apoptosis levels in the *itchy* mice. This, however, does not hold true at PND 28. Although apoptosis levels are higher than the wild type C57BL/6J mice, caspase 9 cleavage is significantly lower, and there lacks an obvious increase in extrinsic death ligand signaling.

Chapter 6: The Functional Role of Itch in the Testis During Development

6.1 Introduction and Rationale

Much of the progress in deciphering the role of physiological Itch has come from the development of the *itchy* knockout mice and the studies of the late onset autoimmune disease that these animals develop (reviewed in[29]). Itch has been shown to target several important regulators of T cell function, including Notch[44], c-Jun[32], and JunB[43]. The loss of Itch results in a preference for Th2 bias in T cell differentiation, leading to the activation of B cells and the over production and deposition of immunoglobulins into the surrounding tissues[43,44]. The *itchy* mice develop severe dermatitis, leading to the scratching that gave the protein its name, and pulmonary inflammation, which ultimately becomes fatal between 6 and 8 months of ages[29]. Although the role of Itch in T cell function has been well characterized, the tissues distribution of Itch along with its identified targets span a wide array of organs and cell types, suggesting that this E3 ligase may play a role, perhaps even a different one than in the immune system, throughout the body.

A limited number of studies have looked at the role of Itch in the testis[39, 46], while testis development in the *itchy* mice has yet to be examined. Several of the previously identified targets of Itch have also been described in the testis, including the tight junction protein occludin[39], which is expressed by Sertoli cells and is an important factor in the blood-testis barrier. Although this target protein has been identified, the *itchy* mice present a unique tool in the study of Itch in the testis. First, junction studies have historically looked that the importance of specific proteins by either reducing their function or knocking them out. The loss of Itch, however, would theoretically cause an

increase in occludin, therefore making the blood-testis barrier less dynamic. Secondly, the testis is typically thought of as an immune privileged organ, meaning that antigens that are formed after the body learns to recognize "self" are protected by other factors, such as Sertoli cell-expressed Fas ligand and the BTB[48, 63]. The *itchy* mice, however, have been shown to have large immunoglobulin deposits in various organs[44], which may also include the testis, possibly leading to an indirect effect on development from the loss of Itch in the immune system. These two possible interactions will need to be teased apart in future studies in order to determine the importance of Itch during testis development.

This chapter was designed to investigate the possible direct and indirect effects that the loss of Itch has on testis development. Within the testis, previously identified targets of Itch were examined both within the seminiferous tubules and in the interstitial space. None of the established targets appeared to be modified within the testes of the *itchy* mice, however IgG deposits in the interstitial space may suggest an indirect effect from the loss of Itch in the immune system. Although the autoimmune disease is a late onset phenotype, with outward dermatitis symptoms presenting around five months of age[29], spleen weights were significantly increased as early as PND 28, suggesting that protein changes seen in the interstitium may result from invading immune cells rather than from a direct loss of Itch in either the germ cells or Sertoli cells.

6.2 RESULTS

6.2.1 Western blot analysis of previously established Itch substrates reveals no significant increases in the testis.

A very limited number of studies have been done to decipher the role of Itch in the testis[39, 40], but several targets of the E3 ligase have been previously identified in other pathways and organ systems[29]. These protein targets have been identified from a wide range of functional pathways, such as cFLIP in apoptosis, c-Jun in cell signaling, and occludin in cell junctions, and here they were examined for their presence and possible alterations in the testes of wild type C57BL/6J and *itchy* mice (Figure 6.1A). Although the previous observation of disrupted apoptosis suggested a dysregulation in cFLIP levels in the *itchy* mice, cFLIP is only detectable at extremely low levels by western blot analysis in total testicular lysates. Although there are age-dependent changes in other two common targets of Itch, c-Jun and occludin, they do not appear to be significantly increased as expected between the wild type and *itchy* mice (Figures 6.1B-C).

6.2.2 Tissue analysis of previously identified Itch substrates may point to a compensating E3 in the testis or a secondary effect from the loss of Itch

A lack of significant observable changes in the testis led to the evaluation of Itch targets in other organ systems in the *itchy* mice, specifically in the liver, spleen, and kidney (Figure 6.2A). These organs were chosen not only because of their simplicity and availability for evaluation, but also because the spleen is known to be a central organ in the immune system. Interestingly, the apoptotic marker cFLIP was significantly increased in the spleen of the *itchy* mice compared to wild type C57BL/6J mice. This is what we predicted would have occurred in the testis but did not observe (Figure 6.1A). The transcription factor c-Jun also was increased in the spleens of these animals, while the tight junction protein occludin was not found to be present in the spleen. The lack of

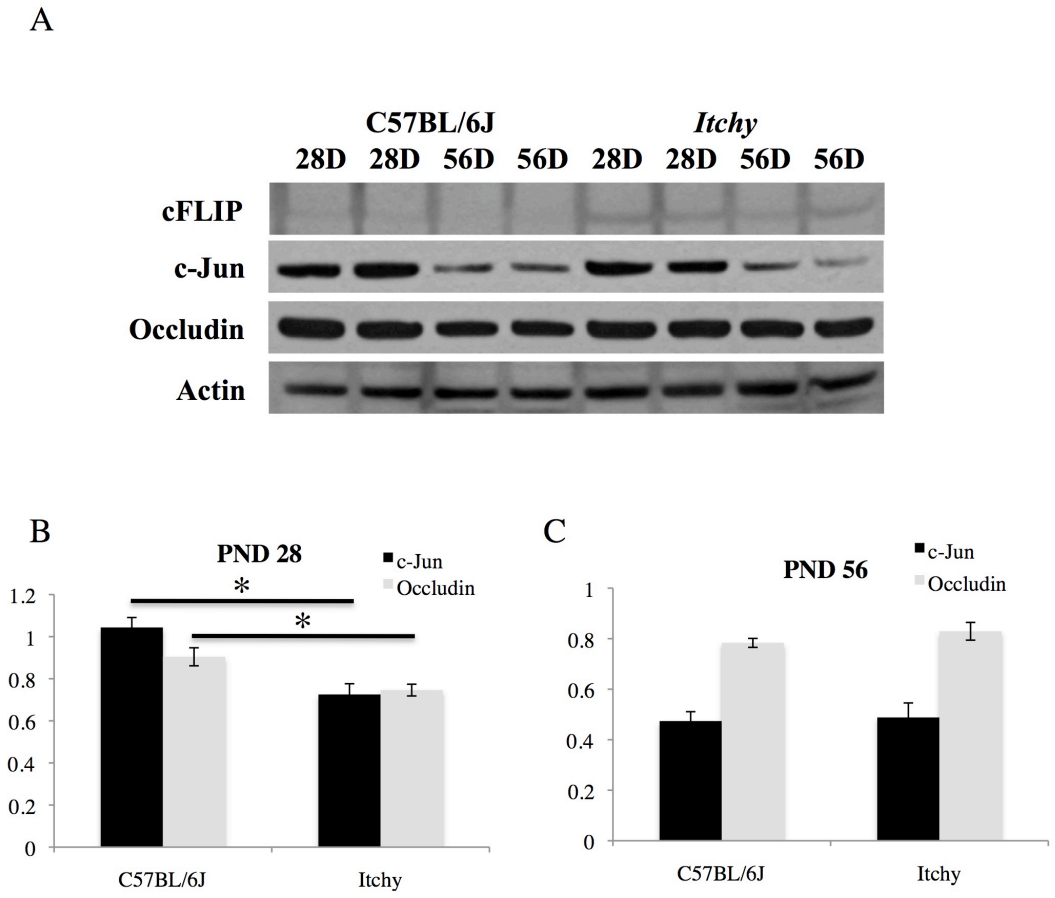


Figure 6.1 Western blot analysis of previously identified Itch targets in C57BL/6J and *itchy* mice at PND 28 and PND 56.

A) Total protein from whole testis tissue was analyzed using western blot analysis and primary antibodies against cFLIP, c-Jun, and occludin. Actin was used as a loading control. Quantification at B) PND 28 and C) PND 56 was performed using ImageJ (NIH). Unexpectedly, cFLIP was only detectable at low levels and no increases were observed in c-Jun and occludin. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

significant changes in the expression of these targets in the testis of the *itchy* mice suggests several possibilities. For example, 1) a compensating E3 ligase may be acting in place of the missing Itch protein, 2) these proteins may not be rapidly translated and degraded in the testis, or 3) they may not be targets of Itch in this organ. An even more provocative hypothesis could be that the loss of Itch in the testis has no effect at all, and in fact it is the overactive immune system that is causing these observable alterations during testis development.

To test the possible influence of the immune system on the testis, a mouse-specific secondary antibody was used in the absence of a primary antibody to measure the IgG levels in the various tissues (Figure 6.2A). Even at PND 28, months before outward symptoms begin to appear, significant IgG deposits can be detected in the liver, spleen, and kidney of the *itchy* mice. Although it typically thought of as an 'immune privileged' organ, immune cells are able to access the interstitial space through blood vessels and can even invade surrounding tubules that are in diseased or damaged states. Interestingly, significant increases in IgG are detected in whole testis preparations from the *itchy* mice compared to their wild type counterparts. A developmental analysis reveals that IgG levels appear to be more significantly different than control at PND 28 than at PND 56, and that this is also true for a seemingly unrelated protein, pERK, and the Fas receptor measured earlier (Figure 6.2B). This data is unexpected as the autoimmune disease is a late onset disorder that gets progressively worse with age, while the higher IgG levels and increased interstitial protein expression suggest that this phenotype may impact the testis in reverse. It was originally hypothesized that because the autoimmune disease intensifies as the animal ages, that the testicular phenotype would behave in a similar fashion and

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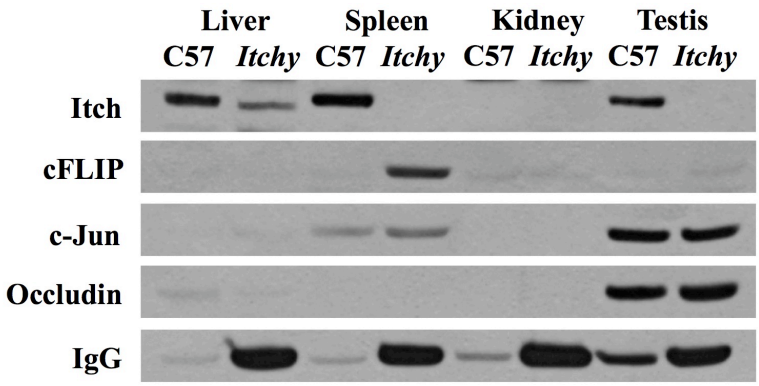

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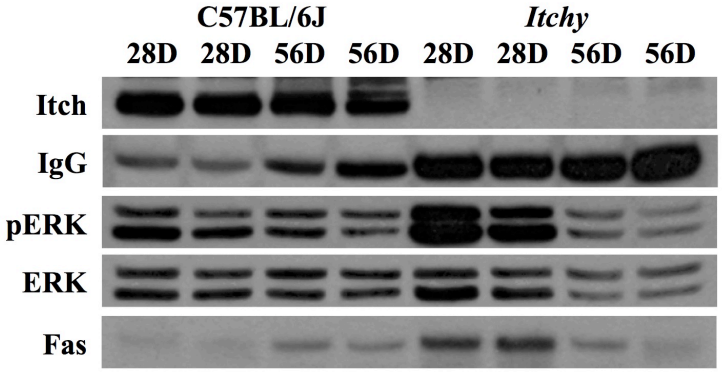

Figure 6.2 Western blot analysis of previously identified Itch targets in various tissues and possible immune interactions in the testis.

Total protein from liver, spleen, kidney, and whole testis was analyzed using western blot analysis and primary antibodies against A) cFLIP, c-Jun, occludin B) pERK, ERK, and Fas. IgG levels were measured using an anti-mouse secondary antibody. *Itchy* mice have an accumulation of IgG in their testis, along with increased Fas and pERK.

worsen with the age of the animal, but in fact the younger PND 28 animals have higher IgG ratios compared to wild type than the PND 56 mice.

6.2.3 *Itchy* mice present with enlarged spleens as early as PND 28, while the typical autoimmune disease remains unseen until much later in development

The physical effects of the loss of Itch present themselves around 5 months of age, while the internal immune phenotype of the *itchy* mice has been detected as early as 8 weeks[29]. However, IgG deposits were measureable and significantly increased in the testes at a much earlier age of PND 28 and remained high at PND 56 (Figure 6.2B). To further investigate the connection between the immune system and the development of the testis, spleen weights were collected at the same ages at which the testes were previously examined (Figure 6.3). At PND 28, *itchy* mice already showed significantly larger spleen sizes than the wild type C57BL/6J mice (*itchy* 0.11±0.01g and C57 0.07±0.01g) and this trend continued at PND 56 (*itchy* 0.11±0.00g and C57 0.08±0.00g, Figure 6.3A). Examination of the spleen to body weight ratios revealed that the *itchy* mice have a ratio almost twice that of the C57BL/6J mice (*itchy* 7.94±0.40 and C57 4.87±0.28g), but that this difference decreases as the animals age and grow in size (*itchy* 5.06±0.18g and C57 3.54±0.22g, Figure 6.3B). These data suggest that although the immune phenotype has been characterized at a much later age, PND 28 *itchy* mice may be suffering detrimental effects on their testicular development due to the loss of Itch in the immune system.

6.2.4 Separation of the seminiferous tubules from the interstitial cells reveals significant changes occurring both within and outside of the seminiferous epithelium

It has long been thought that the testis was a unique organ in that it was immune privileged, meaning it encompassed certain qualities that allowed it avoid the otherwise

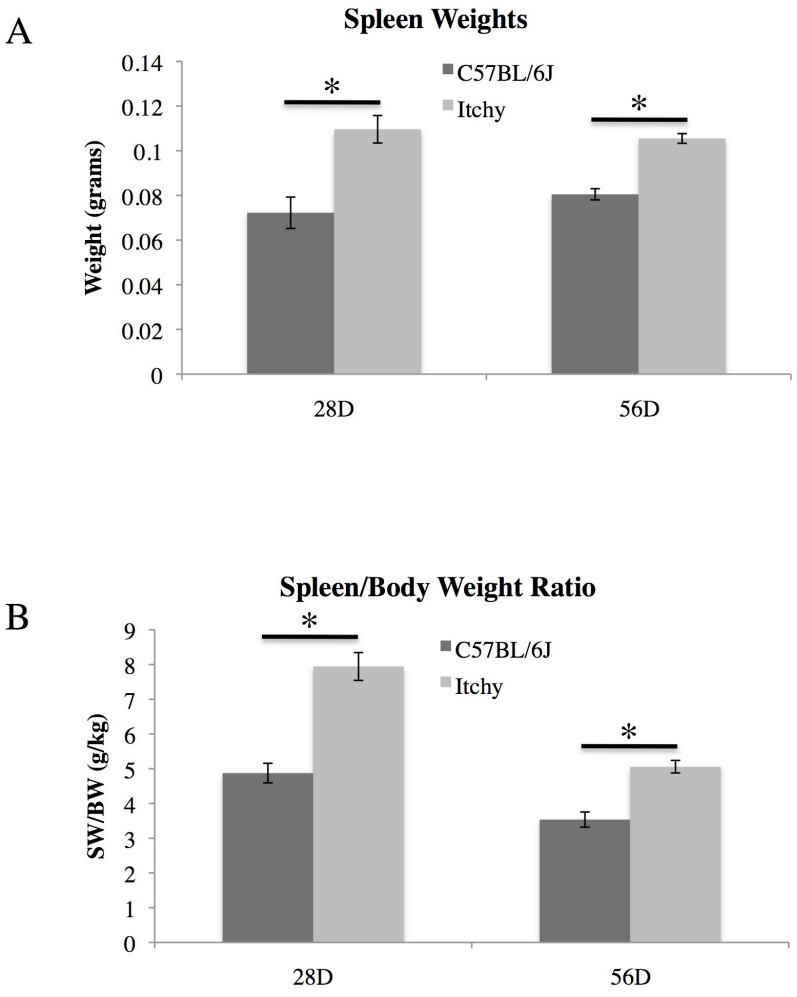

Figure 6.3 Spleen weights and spleen/body weight ratios of C57BL/6J and *itchy* mice.

The A) spleen weights were recorded at PND 28 and PND 56 and compared to B) the body weight of the animal at the same age. *Itchy* mice have significantly larger spleens and spleen/body weight ratios as early as PND 28. Statistical results are expressed as the mean ± SEM and was considered significant (*) if $p < 0.05$.

'self-recognizing' ability of the specialized immune cells. Several mechanisms have been proposed to explain this apparent protection, including death ligands expressed on the surface of Sertoli cells acting as apoptotic initiators and the BTB preventing these immune cells from reaching the developing germ cells, although the exact mechanisms still remain unclear. In order to determine if our previously observed protein changes in the *itchy* mice were originating from an immune cell source, and if so, whether these cells were invading the seminiferous epithelium, tubules were isolated from fresh PND 28 testes and protein expression was examined (Figure 6.4). Luteinizing Hormone Receptor, a Leydig cell-specific marker, was used as a negative control, and was only present in whole testis preparations and not in isolated seminiferous tubule preparations. The IgG deposits that were observed previously in the whole testes of the *itchy* mice were almost completely removed with the removal of the interstitial space, suggesting that the B cells and their secreted immunoglobulins remain outside the tubules and that the BTB remains intact. Interestingly, the measured increases in Fas receptor and the phosporylated form of the ERK signaling kinase also disappeared with the removal of the interstitial space. This indicates that these changes are occurring in cells other than the germ cells or Sertoli cells, pointing to and possibly supporting the hypothesis that the phenotype observed in the *itchy* mice results from an indirect effect of an overactive immune system on a sensitive time period of testis development.

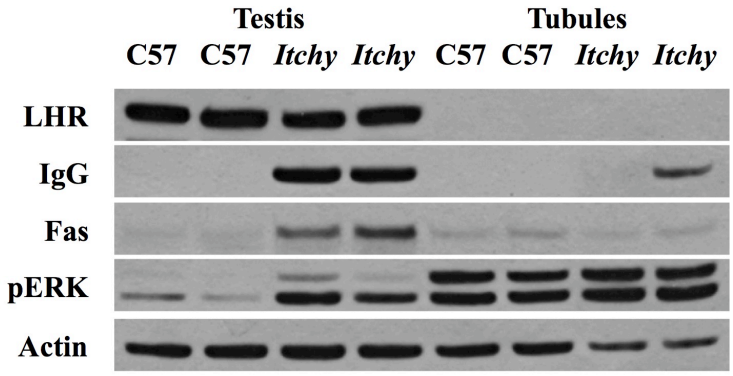

Figure 6.4 Western blot analysis of isolated seminiferous tubules from C57BL/6J and *itchy* mice at PND 28.

Total protein from whole testis and isolated tubules were analyzed using western blot analysis and primary antibodies against LHR, Fas, and pERK. IgG levels were measured using an anti-mouse secondary antibody, and actin was used as a loading control. The observed increases in IgG, Fas, and pERK appear to be localized to the interstitial space.

6.3 DISCUSSION

Itch has been well characterized in the immune system due to the late onset autoimmune disease of the *itchy* mice, and many of the targets of this E3 ligase have already been identified. Important immune targets like c-Jun, which has been well characterized and shown to be upregulated in the T cells of *itchy* mice[43], was not found to be altered in the testis, although it was detectable (Figure 6.1). Occludin, a previously identified testicular Itch substrate[39], is not significantly increased in the *itchy* mice at either PND 28 or PND 56, suggesting a low rate of protein turnover or possibly a compensating E3 ligase which replaces the role of Itch. The anti-apoptotic protein cFLIP, capable of blocking caspase 8 activation and therefore apoptosis, is expressed at very low levels under normal conditions in the testis of wild type mice. Even with the loss of Itch, cFLIP levels remain low at both of the ages examined, which was unexpected given the importance of the FasL death receptor-signaling pathway during spermatogenesis[62].

The lack of any changes in target proteins in the testis was very surprising considering that there were several significant and repeatable alterations throughout development. This led us to look at these targets in several other organs in the *itchy* mice (Figure 6.2A). Both cFLIP and c-Jun appeared to be increased in the spleens of the *itchy* mice, reconfirming the importance of this protein in the immune system. Interestingly, the IgG deposits that were found in the liver, spleen, and kidney of the PND 28 *itchy* mice was also found in the testis, a site that is thought to be protected from this type of invasion. A deeper look revealed that PND 28 *itchy* mice had significantly higher levels of IgG in whole testis preparations than wild type mice, and that these levels remained high at PND 56 (Figure 6.2B). Several other protein changes were also observed at these early time points, suggesting that not only are the *itchy* mice experiencing a testicular

phenotype at PND 28, months before any immune symptoms have been previously reported, but that the immune cells are able to enter and possibly disrupt the testis environment.

Due to the late onset nature of the immune disease, much of the work done with the *itchy* mice has utilized them as adults, 5 to 6 months after birth, while the testicular phenotypes observed here are significantly earlier. Because IgG deposits were measured in the testis at PND 28, spleen weights were also collected to determine the impact of the loss of Itch on the immune system at this young age (Figure 6.3). Unexpectedly, gross spleen weights and spleen to body weight ratios suggest that *itchy* mice are more significantly impacted at PND 28, with greater differences from wild type, than at PND 56. It is unknown whether this has any impact on the health of the animal, but further work is needed to determine if the obviously active immune system is acting at this early developmental stage.

As for the testis, IgG deposits were detected in whole tissues lysates, suggesting that there was an increase in immune cells within the organ, but it was still unclear whether these cells were infiltrating the tubules or remaining in the interstitial space. To help answer this, seminiferous tubules were extracted from testes and protein expression was determined separately from the whole testis and the interstitium (Figure 6.4). As is turns out, the observed IgG deposits, along with the increases in Fas receptor and pERK, were eliminated with the removal of the interstitial space, indicating that these changes were occurring in cells other than the germ cells and Sertoli cells. Although this suggests that the functional role of Itch may lie outside of the tubules, alterations in the interstitium can impact functions within the seminiferous epithelium. This points to an indirect effect from the infiltrating immune cells rather than a direct effect from the loss of Itch in the testis.

Chapter 7: Conclusions and Future Directions

7.1 ITCH ACTS IN AN AGE-DEPENDENT MANNER DURING SPERMATOGENESIS

Spermatogenesis in an intricate process that relies not only on the dividing and developing germ cells, but also on the supportive Sertoli cells that provide nutrition and structure during this important reproductive cycle. It has been recently highlighted that the rapid of turnover of proteins in both cell types serves as a necessary process to aid in various processes such as packaging of the spermatids and uptake of their residual cytoplasm. This study aimed to examine the role of the E3 ubiquitin ligase Itch during spermatogenesis by utilizing the previously developed knockout mouse model *itchy*. Although some of the phenotypes described are subtle, the consistently lower sperm counts and altered germ cell cycle suggest that the loss of Itch has a negative impact on testis development. The significant decreases in litter sizes and observed fetal developmental dysfunctions may point to a more prominent role for Itch in female gamete production or embryonic development, but further work is needed to characterize the role of this ligase in these systems. For now, the alterations observed and described here suggest that Itch acts directly and/or indirectly, most likely both, to influence spermatogenesis, and that its role depends on the developmental stage of the testis.

7.2 ITCH IS REQUIRED DURING FETAL DEVELOPMENT

Most of the knowledge on the functional role of Itch comes from the work using the *itchy* mice and through the study of the late-stage autoimmune disease that they develop. This disease has been very useful for examination of the immune system and Itch targets within T cells, but the role of Itch in other systems or earlier developmental periods remains unknown. This study aimed to look at the overall development and reproductive ability of these *itchy* mice to get a more global perspective on the function

of Itch. Small changes in body and testis weights were observed (Figure 3.1) although it may have been a reflection of the early phases of the autoimmune disease. But more significant were the reproductive changes, where decreased sperm counts (Figure 3.3) and decreased litter sizes (Figure 3.2) suggested that Itch might be playing a role during gamete formation and/or fetus development. Further examination into this question revealed that *itchy* female mice had a fewer number of pups born compared to the number of embryo implantation sites in their uterus (Figure 3.6). This further supported the hypothesis that Itch is required for fetal development, however the fate of these missing embryos was still unknown, and its role in female reproduction was not completely ruled out. Although no developmentally dysfunctional pups were found during the implantation study, the experimental setup may have prevented these findings, as the uteri were not collected until five days after birth. A few *itchy* mothers, however, were sacrificed by CO_2 inhalation one day following the appearance of pups and the uterine horns were examined, and interestingly, some of still had what appeared to be underdeveloped fetuses in their uteri (Figure 7.1). These fetuses were at various stages of development and/or possibly in the process of being reabsorbed, but none of them responded to physical stimulation. This was not observed in the embryo implantation studies, where the female mothers were sacrificed five days after giving birth. This suggests that the females either reabsorbed the less developed fetuses or excreted and disposed of the more the developed one, but the reason for their failure to survive remains unclear. This question is compounded by the fact that the surviving pups, although a small litter size of 4-5 pups, appear healthy and survive to adulthood. One way to test if this is a fetal development or female gamete dysfunction would be to impregnate a wild type female with *itchy* fertilized eggs, allow her to give birth, and then compare the number of implantation sites to the number of pups born. If there are fewer pups than

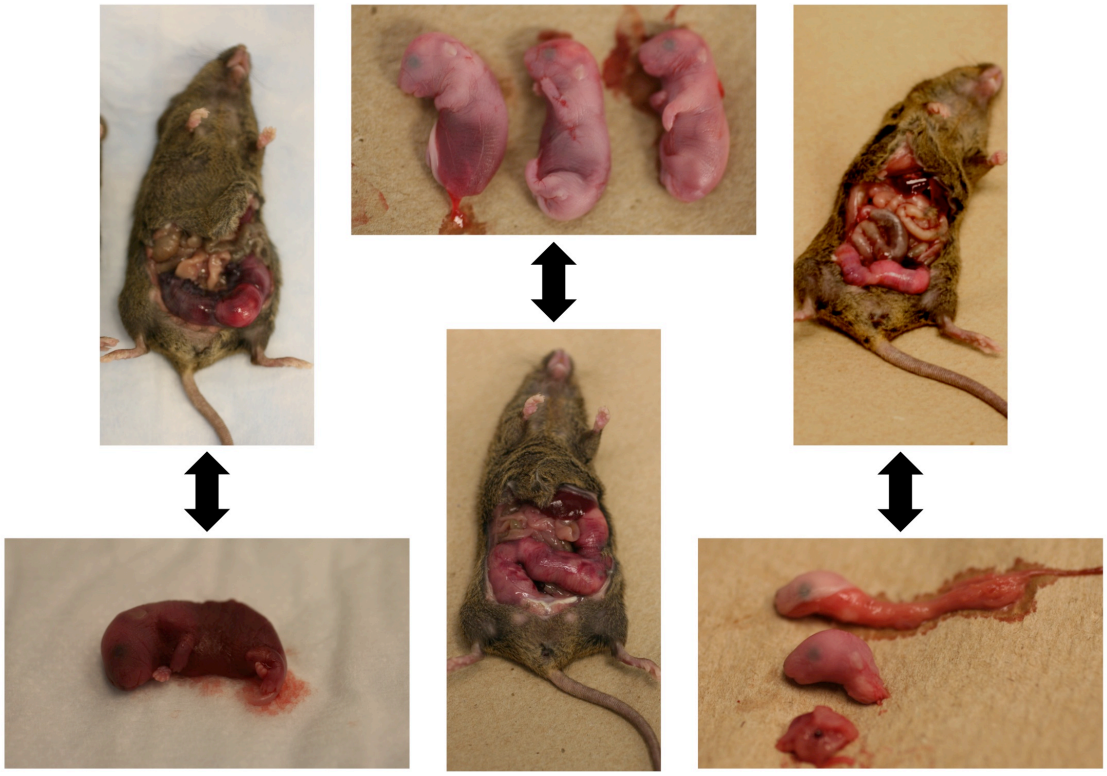

Figure 7.1 Partially developed fetuses retained in the uteri of *itchy* females.

Female *itchy* mice were sacrificed the day after giving birth to a small litter and the contents of their uterine horns were examined. Some of the females still had maldeveloped/partially resorbed fetuses in their uteri, none of which responded to physical stimuli.

implantation sites, then it might suggest a fetal issue, while if the pups and sites are equal, then the decrease in litter size may in fact be a female gamete dysfunction.

7.3 ITCH INFLUENCES THE CELL CYCLE IN DEVELOPING GERM CELLS

Generation of the *itchy* mice has led to a wealth of information about the functional roles of Itch, mostly during later stage development, including a number of various targets and tissue-specific roles. The pathways that this E3 ubiquitin ligase has been linked to vary greatly, including T-cell development and differentiation, apoptosis, cell junction organization, transcriptional regulation, and so on. This study examined the participation of Itch in spermatogenesis, and identified germ cell division as another possible pathway of involvement. At both PND 28 and PND 56, *itchy* mice have a higher number of tubules containing meiotic figures (Figure 4.2) although the younger animals do show a delay in the onset of spermatogenesis (Figure 4.1). A closer look at the seminiferous epithelium revealed another set of unusually dividing cells near the basement membrane that were not present in the C57BL/6J sections (Figure 4.3). These cells were negative for FoxO1, a marker for early spermatogonia cells, suggesting that they are either late stage spermatogonia or early spermatocytes. The presence of these cells along with the observed alterations in meiosis might point to a direct role for Itch in the cell cycle, and some preliminary examinations were done. Western blots targeting the important regulators of the mitotic phase of the cell cycle, including several different cyclins and cyclin-dependent kinases, failed to reveal any significant differences between the *itchy* and wild type mice (data not shown). Unexpectedly, examination of cyclin D, a mediator of the G_o to S transition, appeared to be slightly decreased in the *itchy* mice at PND 28 (Figure 7.2A). Although the importance of this data remains unclear, it led to the discovery of several other proteins that were also disrupted and a possible mechanism

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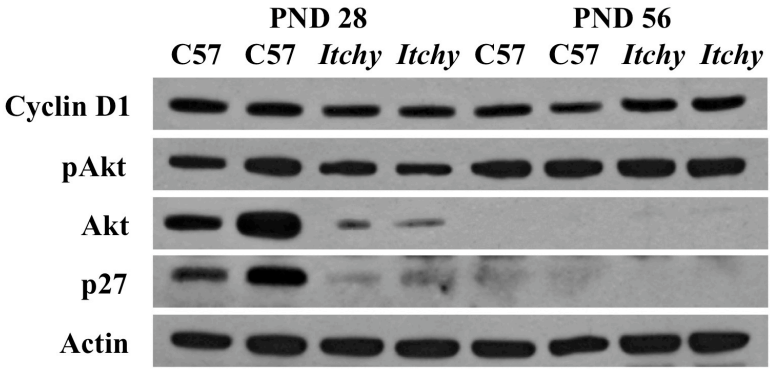

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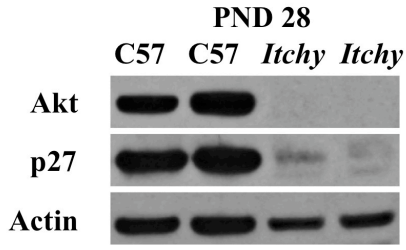

Figure 7.2 Western blot analyses of isolated tubules from C57BL/6J and *itchy* mice at PND 28.

Total protein from isolated tubules was analyzed using western blot analysis and primary antibodies against A) cyclin D, p-Akt (Ser473), Akt, and p27. B) Akt and p27 expression was confirmed using a second pair of control and *itchy* tubules. Actin was used as a loading control.

behind the mitotic phenotype in the *itchy* mice. Akt is an important regulator in the cyclin D pathway and western blots using isolated tubules from PND 28 C57BL/6J and *itchy* mice revealed a significant decrease compared to wild type (Figure 7.2A). Another protein in this pathway, $p27^{kip1}$, shows a similar expression pattern as Akt, and is significantly decreased in the PND 28 *itchy* mice (Figure 7.2A). These data were confirmed using a second pair of PND 28 C56BL/6J and *itchy* mice and similar decreases were observed (Figure 7.2B). A literature search revealed that $p27^{kip1}$ knockout mice had been generated and that the testes had been specifically examined, and interestingly they also presented with an unusual subset of early abnormally dividing cells[64]. The authors explain that these cells are not spermatogonia, but in fact preleptotene spermatocytes which are abnormally entering mitosis rather than undergoing meiosis. The loss of $p27^{kip1}$ and the appearance of these unusual cells near the basement membrane correlate with what is observed in the *itchy* mice, although no known connection between Itch and $p27^{kip1}$ exists. Although the loss of $p27^{kip1}$ may explain the mitotic/meiotic phenotype that is observed in the *itchy* mice, further work is needed to understand how the loss of Itch would result in a similar loss of $p27^{kip1}$.

7.4 THE LOSS OF ITCH DISRUPTS NORMAL GERM CELL APOPTOSIS

Normal levels of apoptosis are required to delete potentially harmful mutations and to maintain the numbers of germ cells to match the supportive capacity of the Sertoli cells. It was originally hypothesized that the *itchy* mice would show a somewhat protected phenotype from this baseline apoptosis due to the removal of the negative regulation of the anti-apoptotic factor cFLIP. Germ cell apoptosis counts revealed, however, that the *itchy* mice in fact had higher levels of baseline apoptosis at both PND 28 and PND 56. Further examination into the two major pathways of apoptosis revealed

some differences between the ages, with increases observed in Fas receptor at PND 28 (Figure 5.3) while increased cleaved caspase 9-positive cells were observed at PND 56 (Figure 5.4). These discrepancies along with the previously observed data suggest that Itch may play an age-dependent role in the testis, perhaps more prominently in germ cell apoptosis, although it remains elusive. As one method to search for possible new and age-dependent targets, total gene expression microarray analysis and 2-dimensional gel electrophoresis were performed using whole testes from PND 28 and PND 56 C57BL/6J and *itchy* mice. Interestingly, the gene expression profile seemed to support an age-dependent role for Itch in the testis (Figure 7.3). At PND 28, 40 genes were differentially expressed (>4 fold) in the *itchy* mice compared to wild type C57BL/6J mice, with 2 increased and 38 decreased. However, at PND 56, 53 genes were differentially expressed (>4 fold) in the *itchy* mice, with 45 of the genes increased and only 8 decreased. This switch from a decrease in gene expression at PND 28 to and increase in gene expression at PND 56, and the fact that only a couple unrelated genes are found on each list, supports the hypothesis that Itch acts in an age-dependent manner during spermatogenesis. The functional groups of these genes also seem to be different depending on the age, with the genes at PND 28 related to cell structure (Col3a1, Krt19, Dpt, etc.) and junctions (Cldn2, Mmp2, etc.) (Figure 7.4) while at PND 56 the focus become more on the immune related genes (Defbs, Cd74, Cd52) (Figure 7.5). There are only a few genes that appear on both lists other than *itch* and the neighboring *agouti* gene, including Romo1, and important mediator in reactive oxygen species (ROS) disposal. This may prove to be an interesting lead, as Itch has recently been identified to target TXNIP, a regulatory protein that functions during oxidative stress[65], for proteasomal degradation. ROS has also been shown to induce germ cell apoptosis[66] and may be responsible for the increased levels observed in the *itchy* mice. In support of this,

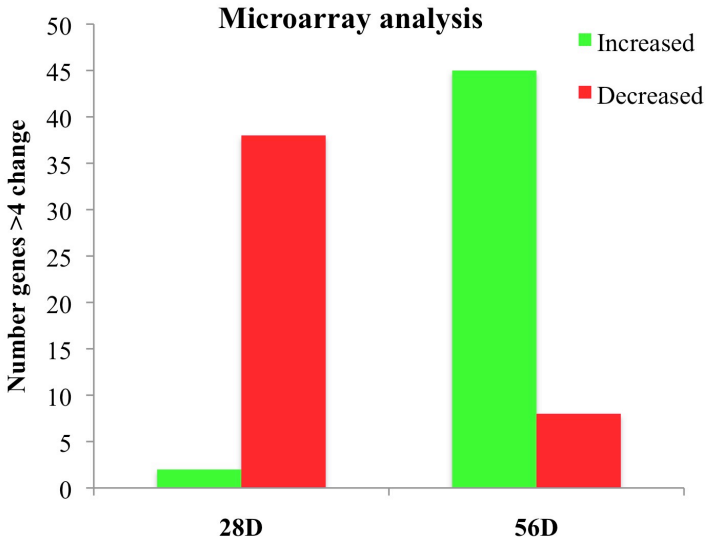

Figure 7.3 Microarray data detailing the number of differences in gene expression of the *itchy* versus C57BL/6J mice

Whole testes were collected at PND 28 and PND 56 and sent to Phalanx Biotech Group (CA) for microarray analysis, and the number of genes in the *itchy* gene profile that differed from C57BL/6J mice by factor of 4 were quantified. A significant number of genes were decreased at PND 28, but this changed at PND 56, where most of the changes were increased.

Ratio (I/C)	Gene	Description
5.257	Romo1	reactive oxygen species modulator 1
4.733	6030422M02Rik	RIKEN cDNA 6030422M02 gene
-3.914	Gnas	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus
-3.927	Ghr	growth hormone receptor
-3.933	Calml4	calmodulin-like 4
-3.977	Col3a1	collagen, type III, alpha 1
-3.984	Cldn2	claudin 2
-3.987	LOC100047146|Gm5840	hypothetical protein LOC100047146|predicted gene 5840
-3.991	Pi15	peptidase inhibitor 15
-4.027	Nnat	neuronatin
-4.036	Krt19	keratin 19
-4.220	Dpt	dermatopontin
-4.307	Fmod	fibromodulin
-4.386	Mmp2	matrix metallopeptidase 2
-4.397	Ahcy	S-adenosylhomocysteine hydrolase
-4.433	Plin4	perilipin 4
-4.458	Cdh16	cadherin 16
-4.493	Col1a2	collagen, type I, alpha 2
-4.535	Cmah	cytidine monophospho-N-acetylneuraminic acid hydroxylase
-4.583	Iyd	iodotyrosine deiodinase
-4.615	1500015O10Rik	RIKEN cDNA 1500015O10 gene
-4.914	Fam47e	family with sequence similarity 47, member E
-5.179	Ggt7	gamma-glutamyltransferase 7
-5.290	Gpx3	glutathione peroxidase 3
-5.336	Pck1	phosphoenolpyruvate carboxykinase 1, cytosolic
-5.438	Aqp1	aquaporin 1
-5.484	Ccdc153	coiled-coil domain containing 153
-5.494	Igfbp5	insulin-like growth factor binding protein 5
-5.946	Itch	itchy, E3 ubiquitin protein ligase
-6.126	Calml3	calmodulin-like 3
-6.229	Lpl	lipoprotein lipase
-6.679	Gpr64	G protein-coupled receptor 64
-7.105	Cidec	cell death-inducing DFFA-like effector c
-7.140	Cdk5r1	cyclin-dependent kinase 5, regulatory subunit 1 (p35)
-7.698	Lum	lumican
-8.001	Cyp2e1	cytochrome P450, family 2, subfamily e, polypeptide 1
-9.402	Hp	haptoglobin
-10.828	Fabp4	fatty acid binding protein 4, adipocyte
-12.924	Car3	carbonic anhydrase 3
-13.188	Cfd	complement factor D (adipsin)

Figure 7.4 Microarray gene expression changes at PND 28

Detailed list of the genes and factor changes measured in the *itchy* mice at PND 28 compared to wild type C57BL/J mice.

Ratio (I/C)	Gene	Description
13.252	Ces5a	carboxylesterase 5A
13.217	Cd52	CD52 antigen
12.818	Spink8	serine peptidase inhibitor, Kazal type 8
10.994	Defb37	defensin beta 37
10.591	Adam7	a disintegrin and metallopeptidase domain 7
10.372	Defb38	defensin beta 38
10.361	Npy	neuropeptide Y
8.956	Spint3	serine peptidase inhibitor, Kunitz type, 3
8.678	Defb2	defensin beta 2
8.615	Ceacam10	carcinoembryonic antigen-related cell adhesion molecule 10
8.575	Cuzd1	CUB and zona pellucida-like domains 1
8.508	Wfdc13	WAP four-disulfide core domain 13
8.173	BC048679	cDNA sequence BC048679
7.657	Acbd7	acyl-Coenzyme A binding domain containing 7
7.333	Ccl8lLOC100503254	chemokine (C-C motif) ligand 8lc-C motif chemokine 8-like
7.312	Peg12	paternally expressed 12
7.265	Gmps	guanine monophosphate synthetase
6.672	Gpx3	glutathione peroxidase 3
6.571	Cwh43	cell wall biogenesis 43 C-terminal homolog (S. cerevisiae)
6.460	Romo1	reactive oxygen species modulator 1
6.070	Defb22	defensin beta 22
5.993	Ch25h	cholesterol 25-hydroxylase
5.937	Ces5a	carboxylesterase 5A
5.521	Akr1b7	aldo-keto reductase family 1, member B7
5.450	Ltf	lactotransferrin
5.450	Gm7455	predicted gene 7455
5.323	Abcb5	ATP-binding cassette, sub-family B (MDR/TAP), member 5
5.295	Retnla	resistin like alpha
5.243	NA	NA
5.052	Defb26	defensin beta 26
4.854	Akr1c19	aldo-keto reductase family 1, member C19
4.802	Gss	glutathione synthetase
4.741	Ramp1	receptor (calcitonin) activity modifying protein 1
4.696	NA	NA
4.515	Spink13	serine peptidase inhibitor, Kazal type 13
4.481	Retnlg	resistin like gamma
4.477	Sat1	spermidine/spermine N1-acetyl transferase 1
4.276	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)
4.268	Pou3f3	POU domain, class 3, transcription factor 3
4.244	Inmt	indolethylamine N-methyltransferase
4.088	Defb42	defensin beta 42
3.960	Cxcl9	chemokine (C-X-C motif) ligand 9
3.944	Lrp2	low density lipoprotein receptor-related protein 2
3.929	Rprm	reprimo, TP53 dependent G2 arrest mediator candidate
3.902	Car4	carbonic anhydrase 4
-4.095	a	nonagouti
-4.110	Ahcy	S-adenosylhomocysteine hydrolase
-5.319	NA	NA
-5.375	NA	NA
-5.992	Itch	itchy, E3 ubiquitin protein ligase
-6.239	Ggt7	gamma-glutamyltransferase 7
-6.522	Myot	myotilin
-11.539	Mfsd4	major facilitator superfamily domain containing 4

Figure 7.5 Microarray gene expression changes at PND 56

Detailed list of the genes and factor changes measured in the *itchy* mice at PND 56 compared to wild type C57BL/J mice.

several other ROS players appear on the gene expression profiles, including glutathione peroxidase and glutathione synthetase. Future work is needed to determine the role of ROS and the possible oxidative stress that may occur in the testes of the *itchy* mice.

Microarrays are extremely useful in that the entire genome can be analyzed and all of the genes of interest, whether changed or not, can be identified. For protein expression changes, two-dimensional (2-D) gel electrophoresis has its limitations due to the requirement for separation of the proteins on a gel and selection of the spots based on concentration differences and availability. However, in the case of the *itchy* mice, differences in protein expression may actually be a more useful tool, as the loss of Itch would theoretically remove a protein that targets other proteins for degradation. So as a preliminary examination, 2-D gels were performed on whole testes from PND 28 (Figure 6) and PND 56 (Figure 7) C57BL/6J and *itchy* mice. In order to eliminate the variability that comes from running and comparing two separate gels, the C57BL/6J and *itchy* samples were labeled with fluorescent dyes and run on a single gel. The green-labeled Cy3 C57BL/6J proteins appeared green when they were present in higher levels than the *itchy* protein at the same site on the merged gel, and similarly for the red-labeled Cy5 *itchy* proteins (Figure 7.6A & 7.7A). From these gels, 50 spots were identified that showed significant difference between the two genotypes and that could be physically selected from the gel (Figure 7.6B & 7.7B). Due to the cost involved, not all of these spots could be identified by mass spectrometry, but several of them were selected for further investigation (Figure 7.8). Interestingly, several of the selected proteins are mitochondrial in origin, including cytochrome b-c1 complex subunit 1, heterogeneous nuclear ribonucleoprotein H, peptidase, and aldehyde dehydrogenase 2. One identified mitochondrial protein, Diablo, is decreased in the PND 28 *itchy* mice, and has also been linked to intrinsic germ cell apoptosis[67]. This is a pro-apoptotic protein that acts by

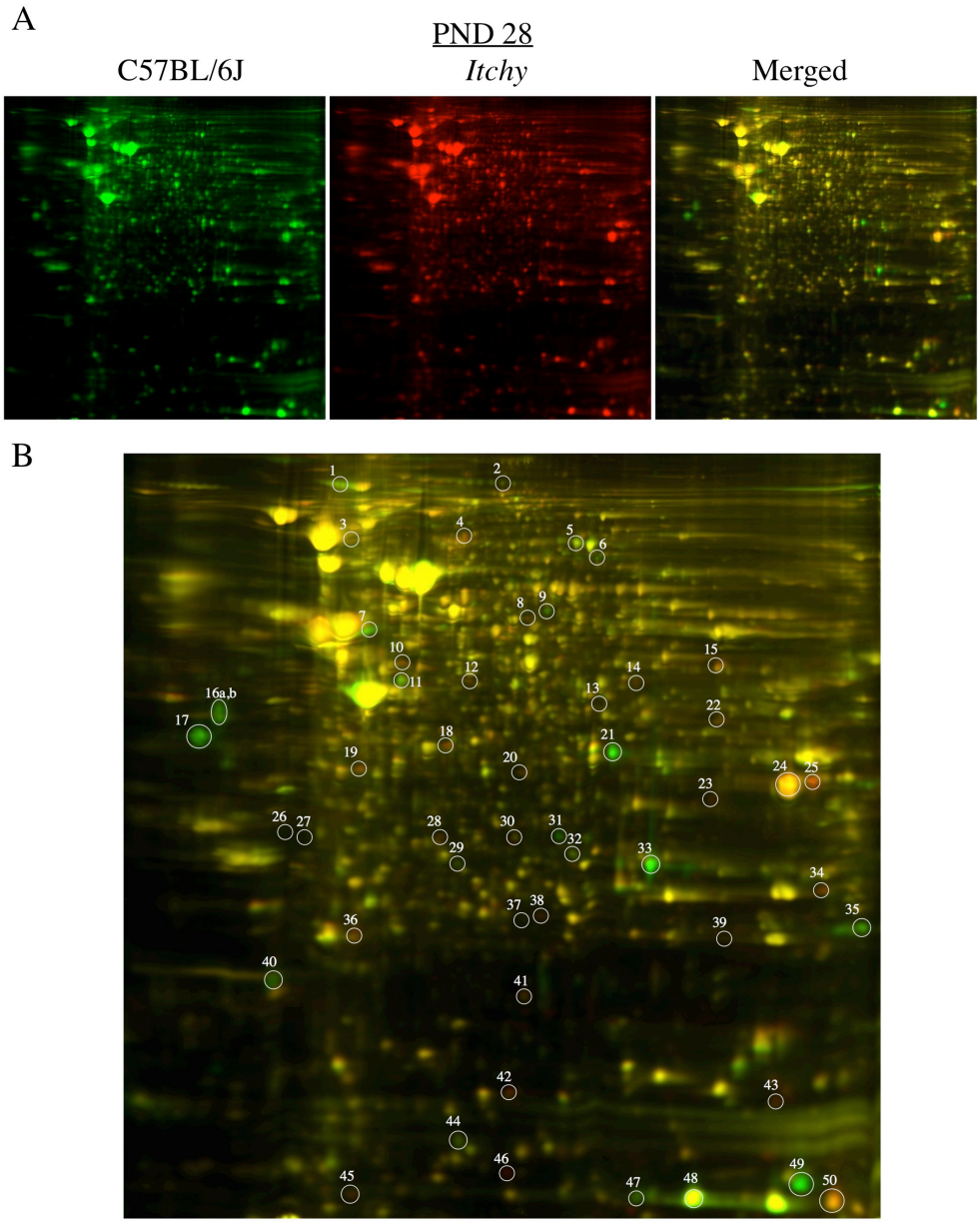

Figure 7.6 2-DIGE images from PND 28 C57BL/6J and *itchy* mice

Whole testes were collected and sent to Applied Biomics, Inc. for 2-D gel analysis. A) C57BL/6J proteins were labeled green, *itchy* proteins red, and when run together, proteins expressed at similar levels appear yellow. B) Spots were identified that showed a difference between the two genotypes and that could be selected from the gel.

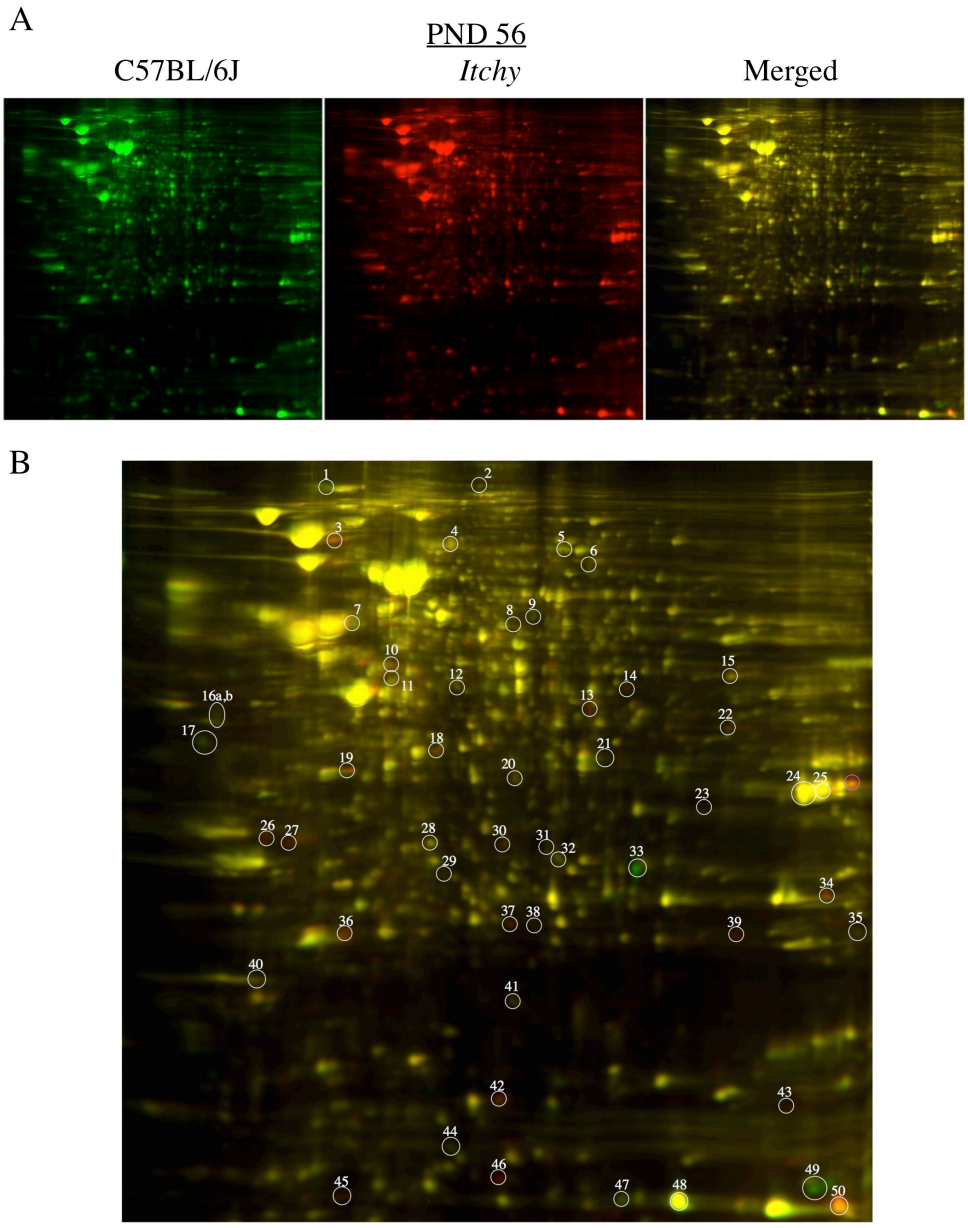

Figure 7.7 2-DIGE images from PND 56 C57BL/6J and *itchy* mice

Whole testes were collected and sent to Applied Biomics, Inc. for 2-D gel analysis. A) C57BL/6J proteins were labeled green, *itchy* proteins red, and when run together, proteins expressed at similar levels appear yellow. B) Spots were identified that showed a difference between the two genotypes and that could be selected from the gel.

inhibiting the anti-apoptotic inhibitor of apoptosis proteins (IAPs) from blocking caspase 9 activation. Therefore, the decrease in Diablo measured in the 2-D gel at PND 28 would support the observed decrease in caspase 9 cleavage by immunohistochemistry (Figure 5.4). Also found on this list of identified proteins are a few related to the oxidative stress pathway, which was previously identified as a possible target pathway for the E3 ligase Itch. Superoxide Dismutase 1 (SOD1), which plays an essential role in protecting cells from dangerous oxygen radicals[67], and aldose reductase, a oxidative stress response gene[68, 69], are both increased in the *itchy* mice, further supporting the link between Itch and the oxidative stress pathway.

7.5 *ITCHY* MICE ARE COMPOUNDINGLY INJURED BY THEIR OVERACTIVE IMMUNE SYSTEM ALONG WITH THE LOSS OF ITCH IN THE TESTES

There is certainly no question that the most important and vital role that Itch plays is in the immune system, both early in life and throughout development, and this is confirmed by the fact that the loss of Itch in the C57BL/6J mouse proves to be fatal. The use of the *itchy* mice as a model system, though, has allowed for other more complex questions to be asked, such as the importance of Itch in other tissues and organ and the effects of a hyperactive immune system on other sensitive organ systems. This study originally set out to examine the role of Itch in the testis by examining the reproductive phenotype of the *itchy* mice and the levels of several previously identified targets. But the lack of significant changes in several known Itch targets, the IgG deposits and location within in testis, and the early age of onset of these observations, led us to consider the possible role of the immune system on the immune-privileged testis. To further tease apart the direct loss of Itch in the testis and the indirect effect of the overactive immune system, the Cre Lox recombination system will be used to remove the influence of the immune system while retaining the loss of Itch in the testis. This method utilizes the site-

Spot	Protein ID	PND 28	PND 56
6	putative ATP-dependent RNA helicase Pl10 [Mus musculus]	-2.71	-1.15
8	Integrator complex subunit 11 OS=Mus musculus GN=Cpsf3l PE=2 SV=1	2.65	1.4
9	carboxylesterase MH1 [Mus musculus]	-3.63	-1.14
10	cytochrome b-c1 complex subunit 1, mitochondrial precursor [Mus musculus]	1.67	1.51
11	creatine kinase B-type [Mus musculus]	-2.54	-1.31
13	RecName: Full=Heat shock-related 70 kDa protein 2; Short=Heat shock protein 70.2	-1.09	3.1
16a	tropomyosin alpha-1 chain isoform 2 [Mus musculus]	-5.31	-1.24
16b	tropomyosin alpha-1 chain isoform 2 [Mus musculus]	-3.72	-1.09
17	beta-tropomyosin [Mus musculus]	-8.7	-1.46
18	L-lactate dehydrogenase B chain [Mus musculus]	1.92	2.06
20	phosphoribosyl pyrophosphate synthetase 1-like 1 [Mus musculus]	1.52	1.16
21	heterogeneous nuclear ribonucleoprotein H [Mus musculus]	-8.57	-1.25
23	voltage-dependent anion-selective channel protein 2 [Mus musculus]	3.1	-1.02
25	L-lactate dehydrogenase C chain [Mus musculus]	2.62	1.05
26	Heat shock-related 70 kDa protein 2 OS=Mus musculus GN=Hspa2 PE=1 SV=1	-1.34	3.16
27	put. beta-actin (aa 27-375) [Mus musculus]	-1.32	5.29
28	peroxiredoxin 4 [Mus musculus]	1.56	1.09
31	carbonic anhydrase 3, isoform CRA_b [Mus musculus]	-6.11	-1.06
33	carbonic anhydrase 3 [Mus musculus]	-10.33	-13.53
35	Chain A, Modified Glutathione S-Transferase (Pi) Complexed With S (P-Nitrobenzyl)glutathione	-4.1	-1.12
37	serine/arginine-rich splicing factor 10 isoform 1 [Homo sapiens]	1.15	2.15
40	diablo homolog, mitochondrial precursor [Mus musculus]	-2.38	-1.01
41	RIKEN cDNA 1700037H04, isoform CRA_d [Mus musculus]	1.69	1.1
42	Chain A, Mouse Sod1	2.21	2.38
43	PREDICTED: peptidyl-prolyl cis-trans isomerase A-like [Mus musculus]	2.51	-1.29
46	aldose reductase-related protein 1 [Mus musculus]	5.63	4.71
47	hemoglobin subunit beta-1 [Mus musculus]	-2.84	-1.8
48	Chain B, Chimeric HumanMOUSE CARBONMONOXY HEMOGLOBIN (HUMAN ZETA2 Mouse Beta2)	-2.65	-1.59
54	peptidase (mitochondrial processing) alpha [Mus musculus]	1.4	1.33
55	aldehyde dehydrogenase 2, mitochondrial, isoform CRA_a [Mus musculus]	1.46	1.43

Figure 7.8 Selected and identified spots on the 2-D gel analysis and their fold changes

Detailed list of the selected proteins and factor changes measured in the *itchy* mice at PND 28 and PND 56 compared to wild type C57BL/J mice.

specific recombinase Cre, which when expressed with a pair of LoxP sites surrounding the gene of interest, cuts the DNA, causing deletion or inversion of the gene. By driving expression of the Cre recombinase from a tissue or temporal-specific promotor, knockout of the gene can be conditional and specific. C57BL/6J mice with the Itch gene flanked by two LoxP sites (floxed) were generated by Dr. Lydia Matesic at the University of South Carolina. For Sertoli cell-specific expression, Cre recombinase will be expressed from the anti-Müllerian hormone (AMH) promotor, and 129.FVB-Tg(AMH-cre) mice have been purchased from Jackson Labs (Figure 7.9A). Sertoli cell-specific expression will be examined first due to the observed loss of $p27^{kip1}$ and its Sertoli cell localization[64], however subtype-specific germ cell cre mice are also commercially available (Jackson Labs). These mice are kept as hemizgotes, as two copies of the Cre recombinase can be toxic. For the initial F_0 cross, an AMH-Cre hemizygous will be mated with a LoxP-Itch homozygous, producing offspring that are all hemizygous for LoxP and theoretically half that are hemizygous for Cre. These F_1 double hemizygotes will then be crossed to a second set of LoxP-Itch homozygous, generating four sets of genetic offspring, one experimental and three control (Figure 7.9B). Of these F_2 pups, the male hemizygous Cre, homozygous LoxP will lack Itch expression in Sertoli cells, but should still express Itch throughout the rest of the testis and the body, and should have a normal functioning immune system. If the testicular phenotype is similar to that of the *itchy* mice, this would suggest that Itch plays an important role in Sertoli cells and that the observed abnormalities are in fact due to the direct loss of Itch in the testis. However, if these F2 mice fail to show any of the previously observed phenotypes, this could suggest that Itch is required in a different cell type in the testis, or that the overactive immune system is in fact resulting in the injured reproductive system. It is likely that all of these factors are

working in concert to cause the observed phenotype in the *itchy* mice, and further work will be needed to tease apart each individual cause and effect.

A

129.FVB-Tg(Amh-cre)8815Reb/J)

B

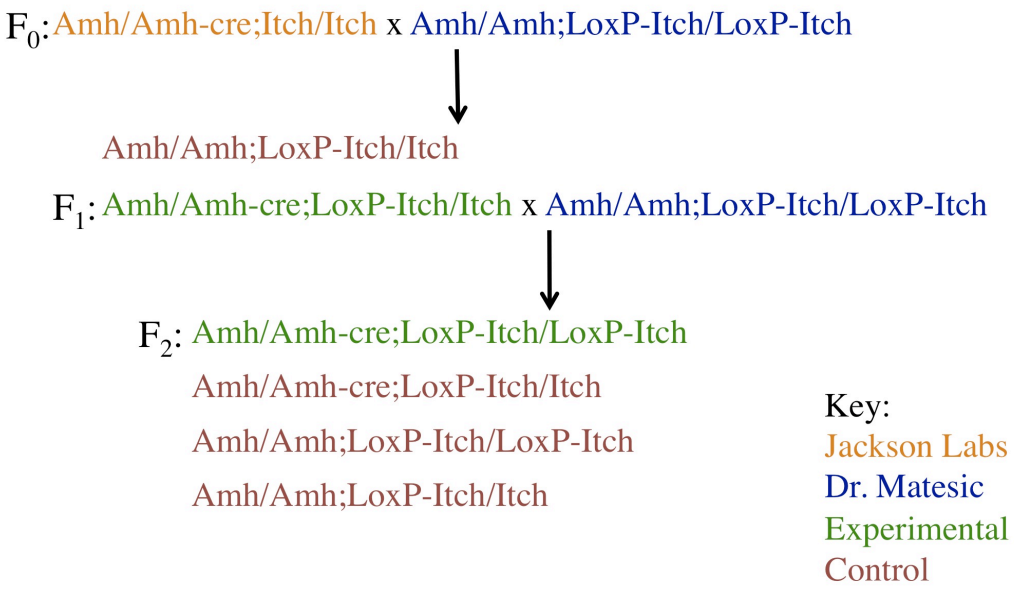

Figure 7.9 Cre-Lox mating scheme for Sertoli cell specific Itch knockout mice

A) Mice with cre expression driven from the anti-Müllerian hormone (AMH) promotor will be mated to B) mice with two LoxP sites on either side of the Itch gene, creating a Sertoli cell-specific Itch knockout. Various colors indicate the source and/or purpose of the mouse strain.

References

1. Hacker, A., et al., *Expression of Sry, the mouse sex determining gene*. Development, 1995. **121**(6): p. 1603-14.
2. Lovell-Badge, R. and A. Hacker, *The molecular genetics of Sry and its role in mammalian sex determination*. Philos Trans R Soc Lond B Biol Sci, 1995. **350**(1333): p. 205-14.
3. Anderson, R., et al., *The onset of germ cell migration in the mouse embryo*. Mech Dev, 2000. **91**(1-2): p. 61-8.
4. Ginsburg, M., M.H. Snow, and A. McLaren, *Primordial germ cells in the mouse embryo during gastrulation*. Development, 1990. **110**(2): p. 521-8.
5. Lawson, K.A. and W.J. Hage, *Clonal analysis of the origin of primordial germ cells in the mouse*. Ciba Found Symp, 1994. **182**: p. 68-84; discussion 84-91.
6. Nel-Themaat, L., et al., *Illuminating testis morphogenesis in the mouse*. J Androl, 2009. **31**(1): p. 5-10.
7. Davidoff, M.S., et al., *Cellular architecture of the lamina propria of human seminiferous tubules*. Cell Tissue Res, 1990. **262**(2): p. 253-61.
8. Diez-Torre, A., et al., *Peritubular myoid cell-derived factors and its potential role in the progression of testicular germ cell tumours*. Int J Androl, 2011. **34**(4 Pt 2): p. e252-64; discussion e264-5.

9. Weber, J.E., et al., *Three-dimensional reconstruction of a rat stage V Sertoli cell: II. Morphometry of Sertoli--Sertoli and Sertoli--germ-cell relationships*. Am J Anat, 1983. **167**(2): p. 163-79.

10. Wong, V. and L.D. Russell, *Three-dimensional reconstruction of a rat stage V Sertoli cell: I. Methods, basic configuration, and dimensions*. Am J Anat, 1983. **167**(2): p. 143-61.

11. Sharpe, R.M., et al., *Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood*. Reproduction, 2003. **125**(6): p. 769-84.

12. Orth, J.M., G.L. Gunsalus, and A.A. Lamperti, *Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development*. Endocrinology, 1988. **122**(3): p. 787-94.

13. Li, N., T. Wang, and D. Han, *Structural, cellular and molecular aspects of immune privilege in the testis*. Front Immunol. **3**: p. 152.

14. de Rooij, D.G. and J.A. Grootegoed, *Spermatogonial stem cells*. Curr Opin Cell Biol, 1998. **10**(6): p. 694-701.

15. de Rooij, D.G. and L.D. Russell, *All you wanted to know about spermatogonia but were afraid to ask*. J Androl, 2000. **21**(6): p. 776-98.

16. Barakat, B., et al., *Activins and inhibins in mammalian testis development: new models, new insights*. Mol Cell Endocrinol. **359**(1-2): p. 66-77.

17. Nakagawa, T., et al., *Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment*. Science. **328**(5974): p. 62-7.

18. Ahmed, E.A. and D.G. de Rooij, *Staging of mouse seminiferous tubule cross-sections*. Methods Mol Biol, 2009. **558**: p. 263-77.

19. Hermo, L., et al., *Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes*. Microsc Res Tech. **73**(4): p. 241-78.

20. Leblond, C.P. and Y. Clermont, *Definition of the stages of the cycle of the seminiferous epithelium in the rat*. Ann N Y Acad Sci, 1952. **55**(4): p. 548-73.

21. Adrain, C. and S.J. Martin, *The mitochondrial apoptosome: a killer unleashed by the cytochrome seas*. Trends Biochem Sci, 2001. **26**(6): p. 390-7.

22. Bratton, S.B. and G.S. Salvesen, *Regulation of the Apaf-1-caspase-9 apoptosome*. J Cell Sci, 2010. **123**(Pt 19): p. 3209-14.

23. French, L.E., et al., *Fas and Fas ligand in embryos and adult mice: ligand expression in several immune-privileged tissues and coexpression in adult tissues characterized by apoptotic cell turnover*. J Cell Biol, 1996. **133**(2): p. 335-43.

24. Fukuzawa, K., et al., *Expression of fas/fas ligand (fasL) and its involvement in infiltrating lymphocytes in hepatocellular carcinoma (HCC)*. J Gastroenterol, 2001. **36**(10): p. 681-8.

25. Koji, T., et al., *Expression of Fas and Fas ligand in normal and ischemia-reperfusion testes: involvement of the Fas system in the induction of germ cell apoptosis in the damaged mouse testis*. Biol Reprod, 2001. **64**(3): p. 946-54.

26. Chinnaiyan, A.M., et al., *FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis*. Cell, 1995. **81**(4): p. 505-12.

27. Bratton, S.B., et al., *Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis*. Exp Cell Res, 2000. **256**(1): p. 27-33.

28. Krueger, A., et al., *Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex*. J Biol Chem, 2001. **276**(23): p. 20633-40.

29. Matesic, L.E., N.G. Copeland, and N.A. Jenkins, *Itchy mice: the identification of a new pathway for the development of autoimmunity*. Curr Top Microbiol Immunol, 2008. **321**: p. 185-200.

30. Peng, J., et al., *A proteomics approach to understanding protein ubiquitination*. Nat Biotechnol, 2003. **21**(8): p. 921-6.

31. Ardley, H.C. and P.A. Robinson, *E3 ubiquitin ligases*. Essays Biochem, 2005. **41**: p. 15-30.

32. Melino, G., et al., *Itch: a HECT-type E3 ligase regulating immunity, skin and cancer*. Cell Death Differ, 2008. **15**(7): p. 1103-12.

33. Sutovsky, P., *Ubiquitin-dependent proteolysis in mammalian spermatogenesis, fertilization, and sperm quality control: killing three birds with one stone*. Microsc Res Tech, 2003. **61**(1): p. 88-102.

34. Rajapurohitam, V., N. Bedard, and S.S. Wing, *Control of ubiquitination of proteins in rat tissues by ubiquitin conjugating enzymes and isopeptidases*. Am J Physiol Endocrinol Metab, 2002. **282**(4): p. E739-45.

35. Kopanja, D., et al., *Cul4A is essential for spermatogenesis and male fertility*. Dev Biol, 2011. **352**(2): p. 278-87.

36. Bedard, N., et al., *Mice lacking the USP2 deubiquitinating enzyme have severe male subfertility associated with defects in fertilization and sperm motility*. Biol Reprod, 2011. **85**(3): p. 594-604.

37. Bedard, N., et al., *Mice lacking the UBC4-testis gene have a delay in postnatal testis development but normal spermatogenesis and fertility*. Mol Cell Biol, 2005. **25**(15): p. 6346-54.

38. Liu, Z., R. Oughtred, and S.S. Wing, *Characterization of E3Histone, a novel testis ubiquitin protein ligase which ubiquitinates histones*. Mol Cell Biol, 2005. **25**(7): p. 2819-31.

39. Lui, W.Y. and W.M. Lee, *cAMP perturbs inter-Sertoli tight junction permeability barrier in vitro via its effect on proteasome-sensitive ubiquitination of occludin*. J Cell Physiol, 2005. **203**(3): p. 564-72.

40. Chihara, M., et al., *Molecular dynamics of the blood-testis barrier components during murine spermatogenesis*. Mol Reprod Dev, 2010. **77**(7): p. 630-9.

41. Hustad, C.M., et al., *Molecular genetic characterization of six recessive viable alleles of the mouse agouti locus*. Genetics, 1995. **140**(1): p. 255-65.

42. Perry, W.L., et al., *The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice*. Nat Genet, 1998. **18**(2): p. 143-6.

43. Fang, D., et al., *Dysregulation of T lymphocyte function in itchy mice: a role for Itch in TH2 differentiation*. Nat Immunol, 2002. **3**(3): p. 281-7.

44. Matesic, L.E., et al., *Itch genetically interacts with Notch1 in a mouse autoimmune disease model*. Hum Mol Genet, 2006. **15**(24): p. 3485-97.

45. Chang, L., et al., *The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover*. Cell, 2006. **124**(3): p. 601-13.

46. Li, M.W., W.M. Lee, and W.Y. Lui, *Expression of Itch in Sertoli cells is controlled via the interaction of E2F1/DP1 complex with E2F and GATA motifs*. Spermatogenesis, 2011. **1**(2): p. 152-158.

47. Meinhardt, A. and M.P. Hedger, *Immunological, paracrine and endocrine aspects of testicular immune privilege*. Mol Cell Endocrinol. **335**(1): p. 60-8.

48. Hedger, M.P., *Immunophysiology and pathology of inflammation in the testis and epididymis*. J Androl, 2011. **32**(6): p. 625-40.

49. Fijak, M. and A. Meinhardt, *The testis in immune privilege*. Immunol Rev, 2006. **213**: p. 66-81.

50. Hedger, M.P., *Macrophages and the immune responsiveness of the testis*. J Reprod Immunol, 2002. **57**(1-2): p. 19-34.

51. Kern, S., et al., *Cytokine secretion by macrophages in the rat testis*. Biol Reprod, 1995. **53**(6): p. 1407-16.

52. Shembade, N., et al., *The E3 ligase Itch negatively regulates inflammatory signaling pathways by controlling the function of the ubiquitin-editing enzyme A20*. Nat Immunol, 2008. **9**(3): p. 254-62.

53. Hudson, D.F., et al., *Centromere protein B null mice are mitotically and meiotically normal but have lower body and testis weights*. J Cell Biol, 1998. **141**(2): p. 309-19.

54. Kumar, T.R., et al., *Male reproductive phenotypes in double mutant mice lacking both FSHbeta and activin receptor IIA*. Endocrinology, 2001. **142**(8): p. 3512-8.

55. Kumar, T.R., et al., *Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility*. Nat Genet, 1997. **15**(2): p. 201-4.

56. Matzuk, M.M., T.R. Kumar, and A. Bradley, *Different phenotypes for mice deficient in either activins or activin receptor type II*. Nature, 1995. **374**(6520): p. 356-60.

57. Schurmann, A., et al., *Reduced sperm count and normal fertility in male mice with targeted disruption of the ADP-ribosylation factor-like 4 (Arl4) gene*. Mol Cell Biol, 2002. **22**(8): p. 2761-8.

58. Walker, W.H., *Molecular mechanisms controlling Sertoli cell proliferation and differentiation*. Endocrinology, 2003. **144**(9): p. 3719-21.

59. Elliott, M.R., et al., *Unexpected requirement for ELMO1 in clearance of apoptotic germ cells in vivo*. Nature. **467**(7313): p. 333-7.

60. Xiong, W., et al., *Gas6 and the Tyro 3 receptor tyrosine kinase subfamily regulate the phagocytic function of Sertoli cells*. Reproduction, 2008. **135**(1): p. 77-87.

61. Rossi, M., et al., *The ubiquitin-protein ligase Itch regulates p73 stability*. EMBO J, 2005. **24**(4): p. 836-48.

62. Lin, Y.C., P.L. Yao, and J.H. Richburg, *FasL gene-deficient mice display a limited disruption in spermatogenesis and inhibition of mono-(2-ethylhexyl) phthalate-induced germ cell apoptosis*. Toxicol Sci, 2010. **114**(2): p. 335-45.

63. Jacobo, P., et al., *Testicular autoimmunity*. Autoimmun Rev, 2011. **10**(4): p. 201-4.

64. Beumer, T.L., et al., *Regulatory role of p27kip1 in the mouse and human testis*. Endocrinology, 1999. **140**(4): p. 1834-40.

65. Zhang, P., et al., *The ubiquitin ligase itch regulates apoptosis by targeting thioredoxin-interacting protein for ubiquitin-dependent degradation*. J Biol Chem, 2010. **285**(12): p. 8869-79.

66. Makker, K., A. Agarwal, and R. Sharma, *Oxidative stress & male infertility*. Indian J Med Res, 2009. **129**(4): p. 357-67.

67. Vera, Y., et al., *Mitochondria-dependent pathway is involved in heat-induced male germ cell death: lessons from mutant mice*. Biol Reprod, 2004. **70**(5): p. 1534-40.

68. Srivastava, S.K., et al., *Aldose reductase inhibition suppresses oxidative stress-induced inflammatory disorders*. Chem Biol Interact. **191**(1-3): p. 330-8.

69. Tammali, R., et al., *Aldose reductase inhibition prevents hypoxia-induced increase in hypoxia-inducible factor-1alpha (HIF-1alpha) and vascular endothelial growth factor (VEGF) by regulating 26 S proteasome-mediated*

protein degradation in human colon cancer cells. J Biol Chem. **286**(27): p. 24089-100.